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14. **ABSTRACT**

Purpose-TRAIL (TNF-related apoptosis inducing ligand) is a protein that induces apoptotic cell death by activating a cascade of cell death caspases. TRAIL is a potential candidate for treatment of prostate cancer. However, it is clear that the majority of prostate cancer cell lines are either insensitive or only partially sensitive to this agent. The purpose of this proposal is to understand the nature of the resistance to this agent and to develop biochemical mechanisms to overcome this inhibition of cell death. Scope-This proposal focuses on TRAIL resistant human prostate cancer cell lines including LNCaP, PC-3 and DU-145. Major Findings-We find that TRAIL induced cell death can be markedly enhanced by the proteasome inhibitor PS-341. The mechanism of action of PS-341 includes increases in the TRAIL receptor, and increases in the BH3 proteins, Bik and Bim. This combination kills cells in the presence Bcl-xL and the absence of Bax but appears to require the Bak protein for activity. Results and Significance-This result suggest that this combination of agents will have significant activity in the clinic. It also suggests that changes in the level of the TRAIL receptor, Dr5, and the protein Bik can be markers of the mechanism of actions of PS-341.

15. SUBJECT TERMS
TRAIL, Apoptosis, AKT, Protein Kinase, Vitamin D

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Introduction

The **purpose** of this grant is to (1) understand the mechanism of resistance of human prostate cancers to TRAIL-induced apoptosis; (2) examine whether the sensitivity of normal tissue to TRAIL depends on the levels of AKT activity; and (3) decipher how cacitriol functions to modulate TRAIL-induced apoptotic killing. The **scope** of the research involves studying normal prostate epithelia in tissue culture, malignant human prostate cancer cell lines, and nude mice with subcutaneous tumors to validate this hypothesis. TRAIL (TNF-related apoptosis inducing ligand) is a protein that trimerizes its receptor and activates caspases to induce apoptotic cell death. We have shown that although some prostate cancer cell lines are extremely sensitive to TRAIL most are either partially or completely resistant. Because TRAIL does not kill normal cells but does kill tumor cells, if resistance could be overcome, this would be a potentially important anticancer agent. We have found that a specific agent PS-341, VELCADE, can overcome this resistance. We have discovered that the mechanism of action of this agent is tied to its ability to regulate the level of BH3 proteins. These findings are clinically important because VELCADE is a novel new clinical agent.

Task 1-

The goal of Task 1 was to create cell lines that would allow us to immunoprecipitate the receptor and examine the proteins that are bound to this complex. The initial proposal was to put into cells a FLAG-tagged receptor that would allow this immunoprecipitation to take place. We have developed an alternative approach. Rather than immunoprecipitating a tagged receptor, we have tagged the hormone. By tagging the TRAIL we do not need to create cell lines. Instead tagged TRAIL can be added to cells and the receptor on the cell surface immunoprecipitated and the DISC brought down with all the bound proteins. A description of the procedure is outlined below. This task involved expressing TRAIL DR5 receptor in prostate cancer cell lines. We used histidine-tagged TRAIL prepared in E. coli added to lysed cells at 1 ug/ml for 20 min on ice. Antihistidine antibody coupled to agarose beads (Sigma) was added to the supernatants which were then incubated with rotation for 2h to overnight at 4C. Beads were washed with TGH buffer (50 mM Hepes pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM EDTA, 1mM EGTA, 0.25 mM PMSF, and protease inhibitors and eluted twice with 100 mM glycine (pH 2.3) for 10 min on ice. Following addition of concentrated SDS-PAGE sample buffer, the eluates were neutralized with 1N NaOH and boiled for 5 min prior to electrophoresis. The SDS-PAGE gel can then be western blotted with antibodies to TRAIL receptors and other components of the Disc, including FADD and caspase 8. An example of this being successfully done is shown in Figure 3 publication 3. This assay enabled us to examine the level of TRAIL receptors.

Task 2-

This task was to examine the binding of specific proteins to the TRAIL receptor in order to quantitate the proteins that may regulate apoptosis. This work has been published in *Cancer Research* and was the basis of a Letter to the Editor (Nesterov A, Kraft AS. In Response 04-4319. Letter to the Editor Can Res 2005; 65:(4)). We find that TRAIL does not kill normal cells or cells immortalized with SV-40 and telomerase, but once cells become transformed by Ras they are then killed by TRAIL.

For these experiments we have used three cell lines, wild type HEK cells, SV-40/Telomerase overexpressor HEK cells, and SV/40/Telomerase/Ras HEK cells. In figure 1 of paper we find that Ras transformed cells undergo apoptosis when treated with TRAIL while the immortalized cells do not.

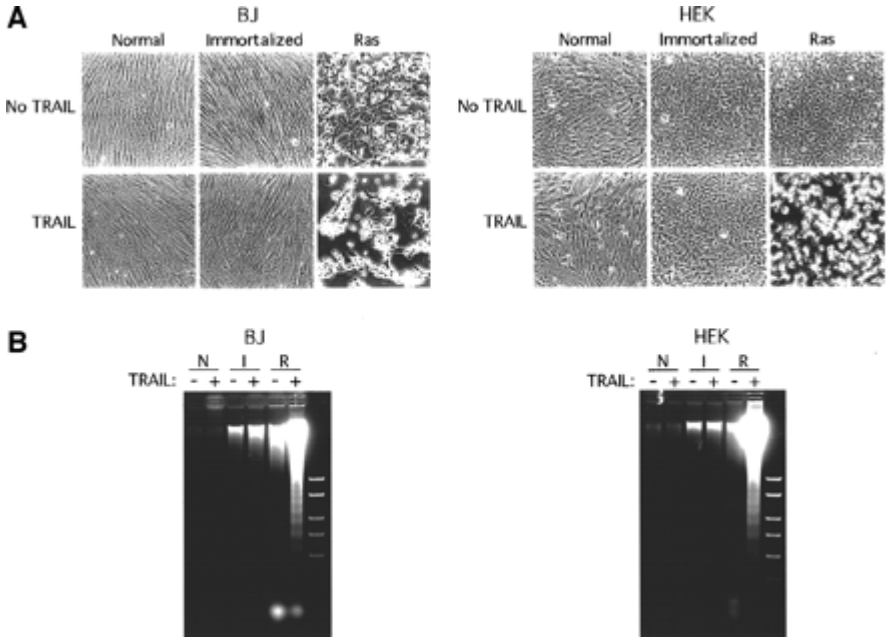

Figure. 1. Transformation of human embryonic kidney cells (*HEK*) and human foreskin fibroblasts (*BJ*) with Ras sensitizes them to tumor necrosis factor-α-related apoptosis-inducing ligand (*TRAIL*)-induced apoptosis. The effects of TRAIL (1 μg/ml) on normal (*N*), immortalized (*I*), or Ras-transformed (*R*) HEK cells and BJ fibroblasts were assessed 72 h after the addition of TRAIL. *A*, the morphology of the plated cells was assessed, and representative photographs are shown. *B*, the cells were harvested, and extracts were processed by DNA laddering assay. DNA laddering was visualized by ethidium bromide staining of 2% agarose gels. Molecular weight markers are shown on the *right*.

In Figure 2, using the technique described in Task 1, we find that Ras transformed cells have increased caspase 8 bound to the Dr5 receptors. We find that there is also increased caspase 8 cleavage. In addition Bid and plectin are cleaved as part of this increase in caspase 8 activity.

Using zVAD-fmk pretreatment, an inhibitor of apoptosis, we find that there is more caspase 8 bound to the membrane (see Figure 3 paper 3). To determine what is causing Ras induced apoptosis and the caspase 8 binding we have used inhibitors of Ras farnesylation SCH 66336, PD 98059, and U0126, the later two being MEK inhibitors. We find that all three of these compounds block the ability of Ras to enhance TRAIL induced apoptosis. This data suggests that the MEK activity is needed for effect of Ras on TRAIL killing. Further we are able to demonstrate that MEK Q56P can duplicate this activity.

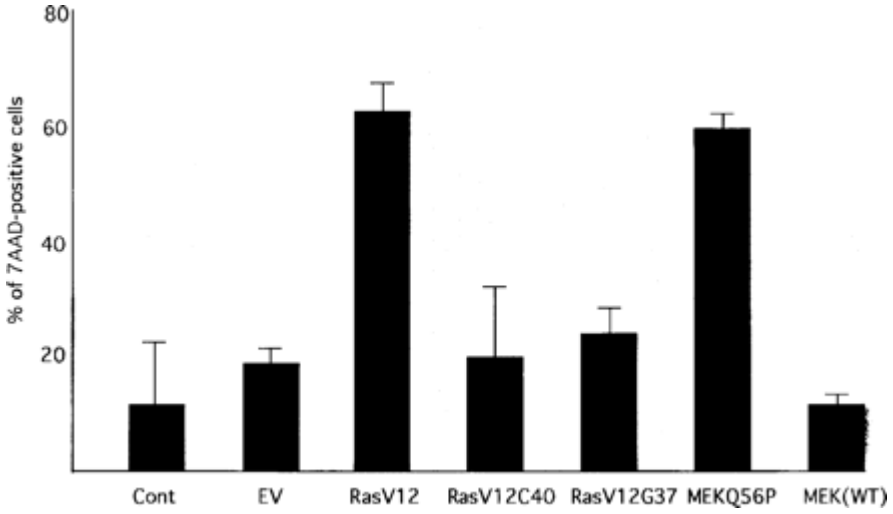

Figure. 2. Proapoptotic effects of Ras are mediated by the mitogen-activated protein kinase pathway. Immortalized human embryonic kidney (HEK) cells were infected with empty retrovirus (*EV*), retroviruses expressing different Ras constructs (as indicated), activated mutant of mitogen-activated protein kinase 1 (*MEK1Q56P*) or wild-type MEK1 [*MEK(WT)*]. Uninfected cells were used as control (*Cont*). The cells were treated with 1 μg/ml tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL) for 48 h. Apoptotic cells were detected by incorporation of 7-aminoactinomycin D (*7AAD*). Data are presented as percentage of 7AAD-positive cells after subtraction of 7AAD uptake by cells not treated with TRAIL. The data shown are the average of three determinations and the SD (*bars*) of the mean.

We do not see any changes in Flip levels, as has been suggested to be important in the literature. We find that blocking the activity of MEK decreases the amount of caspase 8 bound to the Dr5 receptor. We find that constitutively active MEK is sufficient to increase the amount of caspase 8 bound to the receptor. It is clearly the goal of this Task to look at what is bound to the receptor. We have accomplished this Task by determining what is bound to receptors in transformed cells.

Task 3 –

This task examined the regulation of Bid cleavage during the apoptotic process, and the ability of AKT to regulate this enzyme. The results detailed in this paragraph have been published in paper 1. To evaluate the control of Bid cleavage by TRAIL we used both prostate cancer and liver cells. We have added TRAIL (100 ng) to LNCaP cells and seen minor cleavage of caspase 8. We find that the addition of 1ug of the proteasome inhibitor PS-341, VELCADE, markedly increases the level of caspase 8 cleavage. The cells were incubated overnight before extracts were made. Thus BID cleavage is related to both the presence of TRAIL and the proteasome inhibitor. We find that the addition of both agents to HCT116 cells which are Bax -/- cells is still sufficient to induce the cleavage of BID.

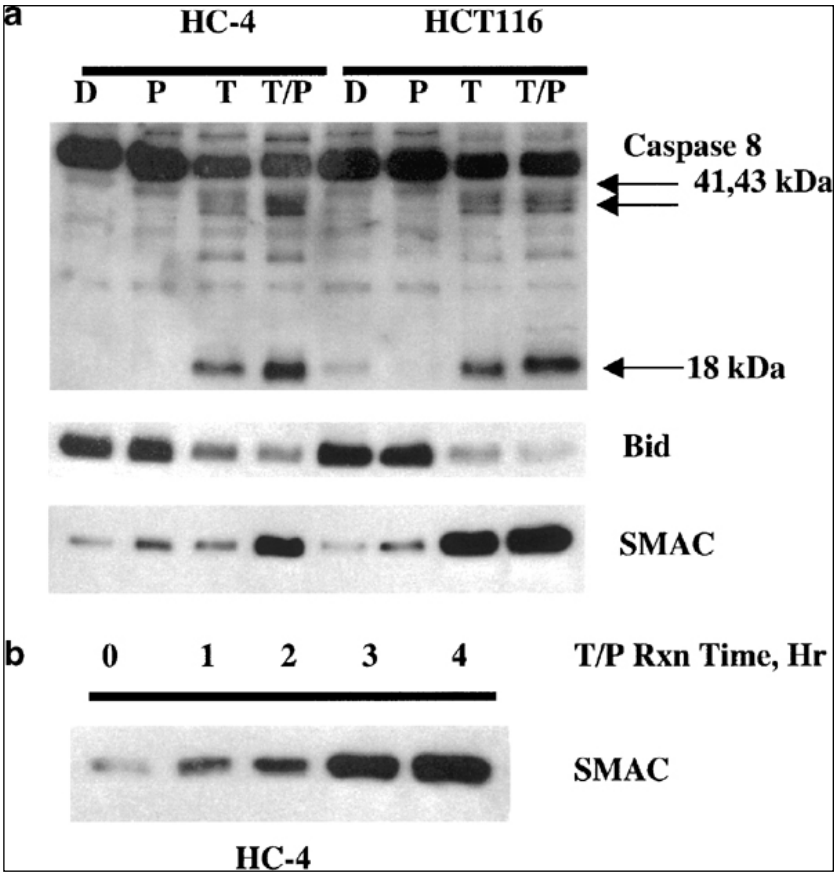

Figure 3. PS-341 enhances the ability of TRA to release SMAC from Bax-deficient HC-4 c (**a**) SMAC is released from HC-4 cells by the combination of TRAIL and PS-341. HC-4 an HCT-116 were treated for 3 h with DMSO (D TRAIL (T) (100 ng/ml), PS-341 (P) (1 M) both (T/P) agents. Extracts of these cells wer run on 12% SDS : PAGE gels and Western blotted with antibodies to the indicated protei (**b**) HC-4 cells rapidly release SMAC after th addition of TRAIL (100 ng/ml and PS-341 (1 M) (T/P). HC-4 cells were treated with both compounds for varying periods of time as shown. Extracts of these cells were treated as (**a**)

We find that PS-341 treatment is associated with increases in Dr5 and Dr4 both at the level of the protein and the mRNA. We find an approximately 8-fold increase in the mRNA and a similar increase in the protein. We find that the protein levels are stabilized by PS-341.

Using an HA-ubiquitin construct we find that the Dr5 receptor is ubiquinatated and that PS-341 increases the level of this ubiquitin.

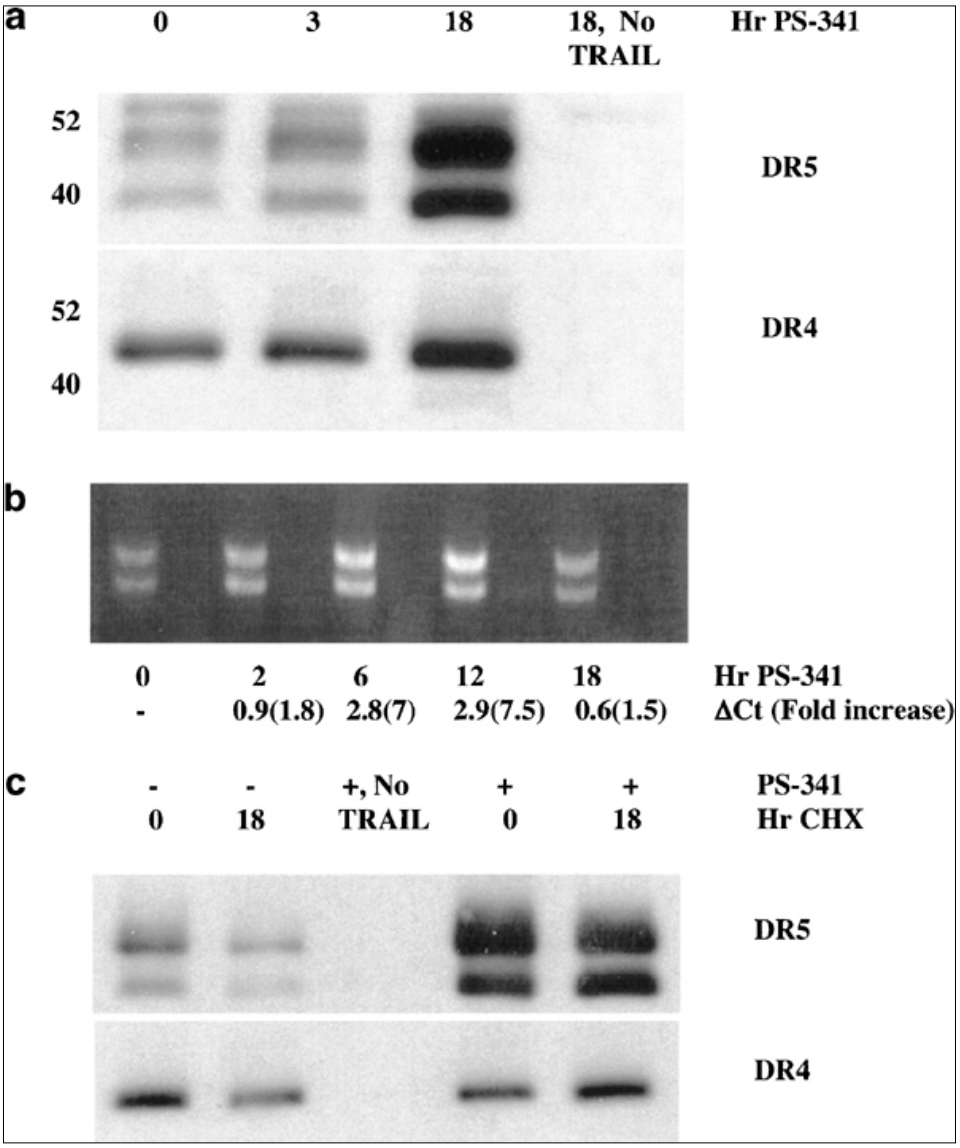

Figure 4. Response of TRAIL receptor protein and mRNA expression to PS-341 treatment. (**a**) PS-341 treatment increases cell surface DR5 and DR4 receptors. HC-4 cells were exposed to 1 μM PS-341 for 0, 3, and 18 h. Cell surface TRAIL receptors were prepared as described in Materials and methods and analyzed by Western blotting for DR4 and DR5 protein. The lane labeled 'No TRAIL' represents cells to which histidine-tagged TRAIL was not added prior to cell lysis and incubation with antihistidine agarose (cf. Materials and methods). (**b**) DR5 transcript levels increase in response to PS-341 treatment. Semiquantitative PCR (gel) and quantitative real-time PCR (Δ Ct) were performed on cDNA prepared from RNA of HC-4 colon cancer cells exposed to PS-341 (1 μM) for various times. ΔCt values (the difference in cycle number at a given threshold during the linear phase of amplification) are relative to control cells; fold increases ($2^{-\Delta Ct}$) are in parentheses. (**c**) PS-341 treatment prevents degradation of DR4 and DR5 protein. HC-4 cells were exposed to vehicle or PS-341 for 6 h. At this point, cycloheximide (50 μM) was added and incubation was continued for an additional 18 h. Total cellular TRAIL receptors were isolated as described in Materials and methods and subjected to Western analysis for DR4 and DR5. The lane labeled 'No TRAIL' is as in (**a**)

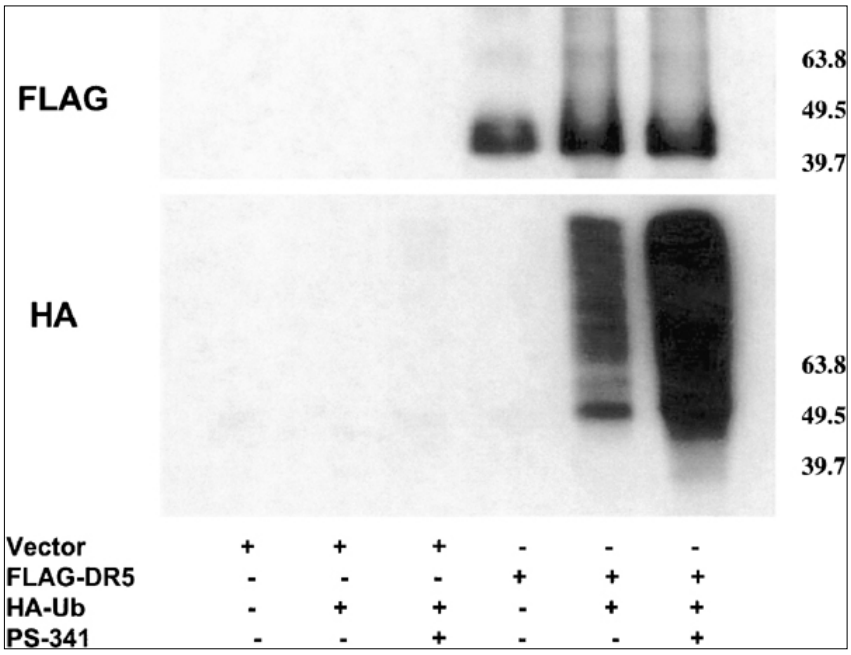

Figure 5. Ubiquitination of DR5. Expression vectors containing FLAG-tagged DR5 (L334N) (FLAG-DR5), HA-tagged ubiquitin (HA-Ub), or empty vector were transfected into 293 T cells in the combinations indicated. After 24 h, PS-341 (1 μM) or vehicle was added and the cultures were incubated overnight. Cell lysates were incubated overnight with anti-FLAG antibody covalently coupled to agarose beads at 4°. Bound material was eluted and prepared for Western blotting as described for DR5 immunoprecipitation. Blots were stained for DR5 (FLAG) or ubiquitin (HA) as indicated

Finally using MEFs that are KO for bax or bak or bax and bak, we find that bak but not bax is needed for the combined death induced by PS-341 and TRAIL. Thus bax -/- cells still undergo death when both agents are added. However, Bak KO cells do not.

Thus, we have found that Bid cleavage can be enhanced by the addition of TRAIL and PS-341. PS-341 increases the levels of TRAIL receptor to enhance BID cleavage. Clearly the ability of cells to die is controlled by Bak. This suggests that PS-341 may be elevating the level of a specific protein that can bind to Bak.

Task 4-

This task involves attempting to understand the role of Akt in this process. Because Akt regulates the NF-kB signal pathway, we have evaluated the role of NF-kB in TRAIL killing. These experiments were done in renal cancer cells because the NF-kB signal transduction pathway is activated in this model. Our results suggest that modulation of the NF-kB pathway does not regulate the ability of TRAIL to kill. For example, if NF-kB is upregulated by treatment with TNFα these cells do not become any more sensitive to TRAIL.

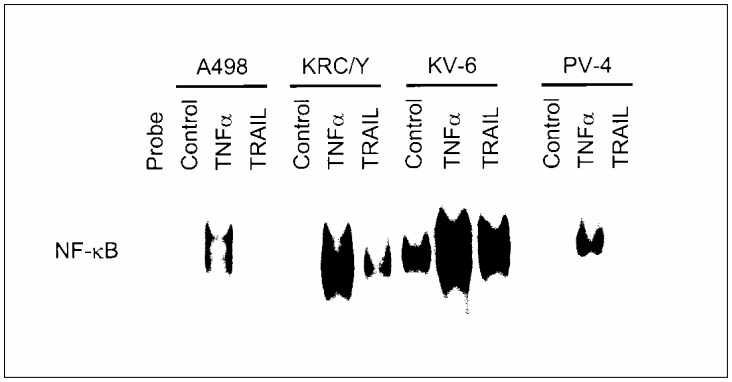

Figure 6. Activation of NF-χB in response to TRAIL. The TRAIL sensitive cell lines A498 and KV-6 and resistant cell lines KRC/Y and PV-4 were either left untreated or treated with 100 ng/ml TNFα or 300 ng/ml TRAIL for 45 minutes. Nuclear extracts were prepared and EMSA was performed using a ^{32}P-labeled NF-χB binding site of the H2-K^{b} MHC class I promoter consensus binding element. A control lane containing labeled- probe in the absence of nuclear extracts demonstrates no mobility shift. Data for PV-4 cells shown was obtained in a separate experiment.

To further evaluate the signaling pathway we have used a dominant negative IkB encoded by an adenovirus. It has been shown by multiple laboratories that a dominant negative IkB will inhibit NF-kB signaling. We have infected with the Ad-mIkB and then examined the sensitivity of the cells to TRAIL killing. We find that inhibiting NF-kB does not affect the ability of TRAIL to kill. Likewise, we do not find that there is any increased sensitivity to TRAIL as a killing agent.

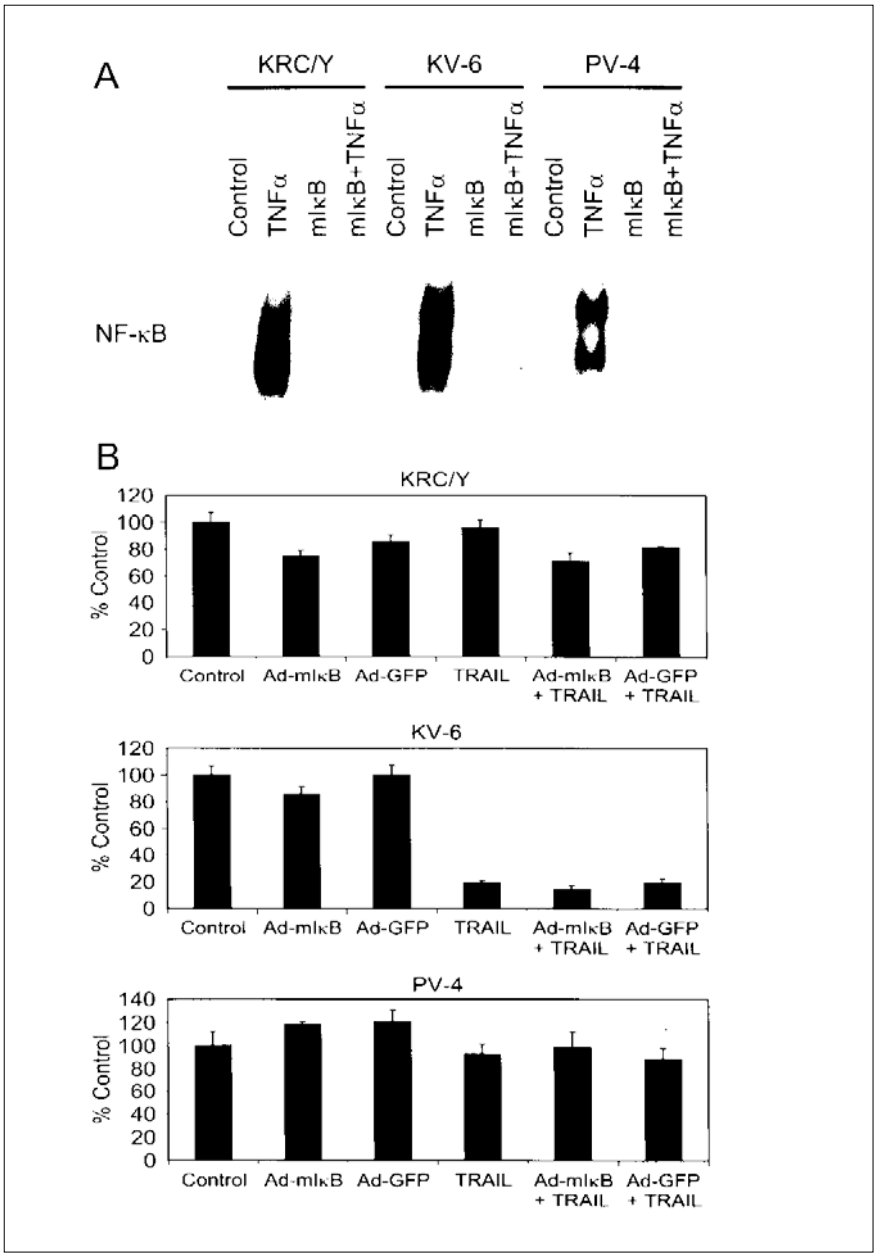

Figure 7. Inhibition of NF-χB using Ad-mIχB does not affect TRAIL medicated cytotoxicity in RCC cell lines. A. To determine the effectiveness of Ad-mIχB to inhibit NF-χB activation, KRC/Y, KV-6 and PV-4 cells were left untreated or transduced with Ad-mIχB at a MOI of 20 for 18 h prior to stimulation with 100 ng/ml TNFα. EMSA was performed as in Figure 6 in cells treated with TNFα following transduction with Ad-mIχB (mIχB + TNFα). B. To determine effects of NF-χB inhibition on TRAIL sensitivity in KRC/Y, KV-6 and PV-4 cells, cells were either left untreated or transduced with Ad- mIχB or a control Ad-GFP construct at a MOI of 20. After 18 h cells were either left untreated or treated with 300 ng/ml TRAIL. Cell viability was measured using an MTS based assay 72 h following TRAIL treatment and expressed as percent control relative to untreated cells. Data are expressed as mean +/- SD for triplicate determinations.

Our results suggest that at least one target of Akt, NF-kB is not essential for TRAIL induced cell death.

Task 5-

In this task we examined whether normal prostate epithelial cells are sensitive to TRAIL. We have found that TRAIL application to normal prostate cells induces apoptosis. This induction of apoptosis is enhanced by the addition of cycloheximide. The effect of TRAIL is irrespective of the passage number of the primary cells in culture and is not correlated with the presence or absence of the decoy receptors as determined by PCR. This result cannot be explained by changes in the levels of Flip 1, gamma or delta. We find that cycloheximide increases TRAIL killing of these normal cells. These data are published in Oncogene.

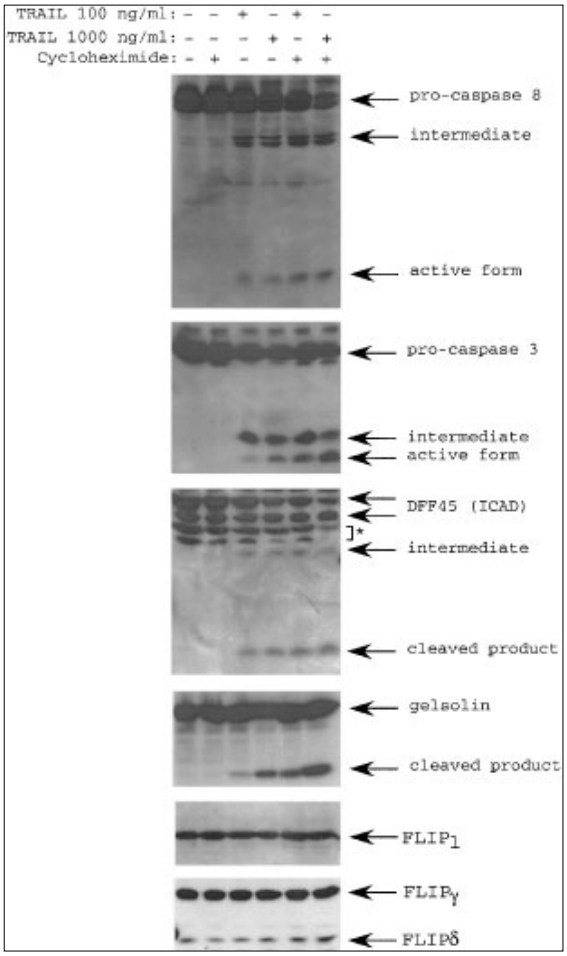

Figure 8. PrEC were pre-treated overnight with 10μM cycloheximide or left untreated. Cultures were then incubated for 6 h with 100 or 1000 ng/ml of TRAIL alone or in combination with cycloheximide. Cell lysates were electrophoresed and the same Western blot was consecutively probed with antibodies specific to caspase 8 (Upstate Biotechnology, Lake Placid, NY, USA), caspase 3 (Transduction Laboratories, Lexington, KY, USA), DFF45 (Zymed Laboratories, So. San Francisco, CA, USA), gelsolin (Sigma, St. Louis, MO, USA), $FLIP_1$ (ABR, Golden, CO, USA) and FLIP [illegible] (Calbiochem, San Diego, CA, USA)

Task 6-

Instead of following up on the initial observation that calcitriol enhanced TRAIL killing we instead made the observation that TRAIL killing is enhanced by PS-341. During the time period of the grant we were not able to accomplish the animal experiments listed in *Task 6, because of availability of reagents.* We first attempted these experiments with PC-3 cells rather than PPC-1. We created sufficient TRAIL for 3 animals. However, the amount of TRAIL needed was underestimated. Because during the grant period neither TRAIL nor PS-341 was FDA approved we could not obtain sufficient collaboration amongst the companies to get these experiments accomplished. We are still attempting to complete these experiments since PS-341 is now marked to the public as VELCADE and TRAIL receptor cytotoxic antibodies are available. This task was designed to discover specific small molecules that enhance TRAIL killing. We have found that PS-341 greatly enhances the killing of prostate cells. This observation is true even when the cell type being studied is insensitive to either PS-341 or TRAIL.

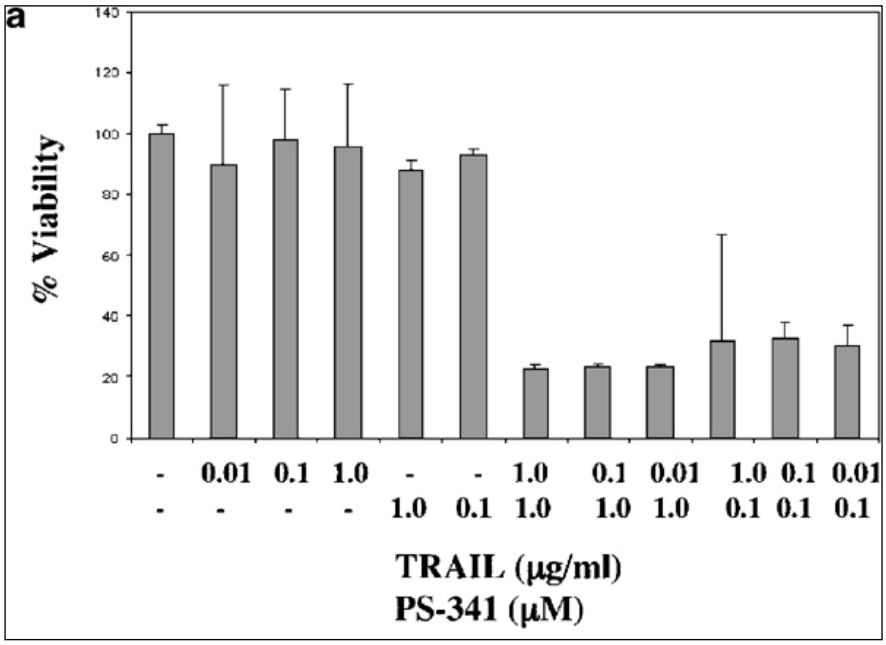

Figure 9. PS-341 treatment markedly enhances TRAIL-induced apoptosis. (**a**) Cell viability assay of cell growth. LNCaP cells were treated with PS-341 or TRAIL in varying amounts overnight and the MTS assay was performed as described in Materials and methods. The s.d. of triplicate determinations is shown. (**b**) Nucleosome ELISA assay of treated LNCaP cells. LNCaP cells were treated overnight with vehicle, PS-341 (1 μM), TRAIL (0.1 μg/ml), or the combination of these two agents. The s.d. of triplicate determinations is shown. (**c**) The effect of PS-341, TRAIL, or both agents on the levels of specific cellular proteins. LNCaP cells were treated as in Figure 1b (D, DMSO; T, TRAIL; P, PS-341, and P/T, PS-341 and TRAIL). Extracts were run on a 10% SDS : PAGE gel, transferred to Immobilon, and Western blotted with antibodies to ubiquitin, $p21^{waf-1}$, c-Myc, and PARP

The addition of the combination of these agents to LNCaP human prostate cancer cells increases the amount of cell death. We do not find that this is secondary to changes in Flip proteins since both the L and S levels are unchanged with PS-341 treatment. The ability of these agents to kill does not appear to depend on caspase 9. We have demonstrated this observation by using mouse embryo fibroblasts that are KO for caspase 9. We also demonstrate that Bak rather than Bax is more important for the killing mediated by these agents (see figure below). The discovery that PS-341 enhances TRAIL killing is important because PS-341 is now a commonly used drug in cancer and is in trial in our center in prostate cancer.

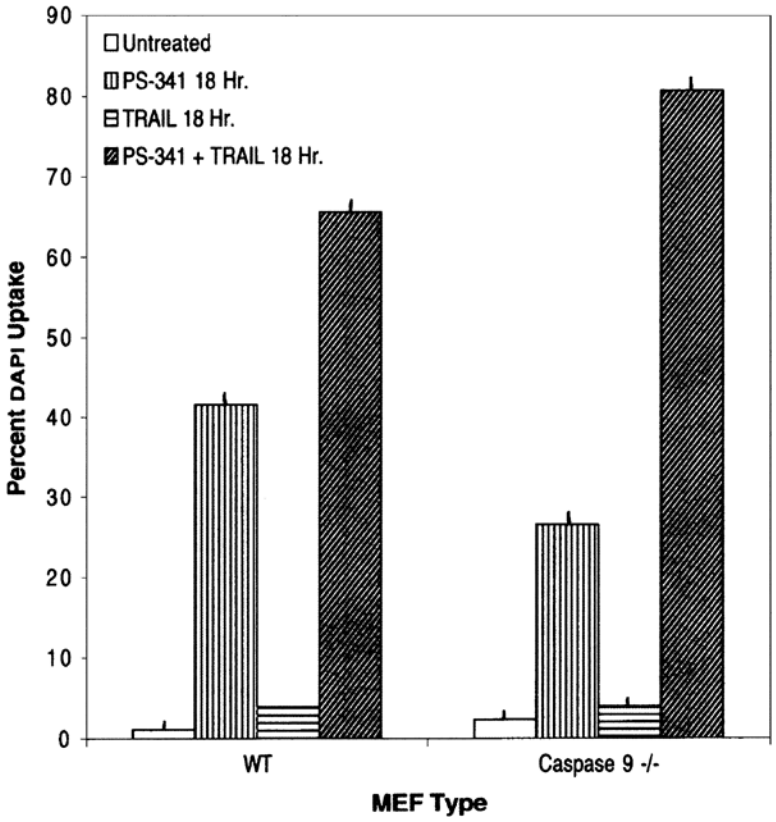

Figure 10. Response of caspase $9^{-/-}$ MEF to PS-341 and TRAIL. Caspase $9^{-/-}$ MEF were exposed to vehicle, 0.1 μM PS-341, 1 μg/ml TRAIL, or the combination for 18 h. Cells were processed for DAPI uptake as described in Materials and methods. Data are expressed as mean and range of two independent experiments

Task 7- In Task 7, since we chose to work with PS-341 rather than calcitriol for both aims 6 and 7, the goals of this aim were focused on how PS-341 works in the cell rather than calcitriol. We examined each of the portions of this Task for PS-341. As described in part a) we evaluated the receptor levels. This was done with the assay that was generated in Task 1. We found that PS-341 treatment of the prostate cancer cells increased the levels of Dr5 8 fold and Dr-4 approximately 3 fold. This increase in receptor was important for the generation of apoptosis. We followed up on part b) of the Task by looking at the downstream signals of PS-341 and TRAIL. We found that the combination caused the cleavage of caspase 8 and BID. This was associated with the release of SMAC from the mitochondria in a time dependent fashion. SMAC release parallels that of cytochrome C, but is easier to accomplish this western. For part c), unlike calcitriol, it is well known that PS-341 inhibits NF-kB degradation. This has been demonstrated in multiple publications and is part of the supposed mechanism of action of this drug and the initial attraction to using this agent. This task attempts to examine in detail the mechanism by which PS-341 enhances TRAIL killing. We have found that PS-341 (Bortezomib) increases the levels of Bik in all cell lines tested. In some cell lines we also found that Bim was increased. We found that knocking out Bim using RNAi did not prevent cell death induced by PS-341.

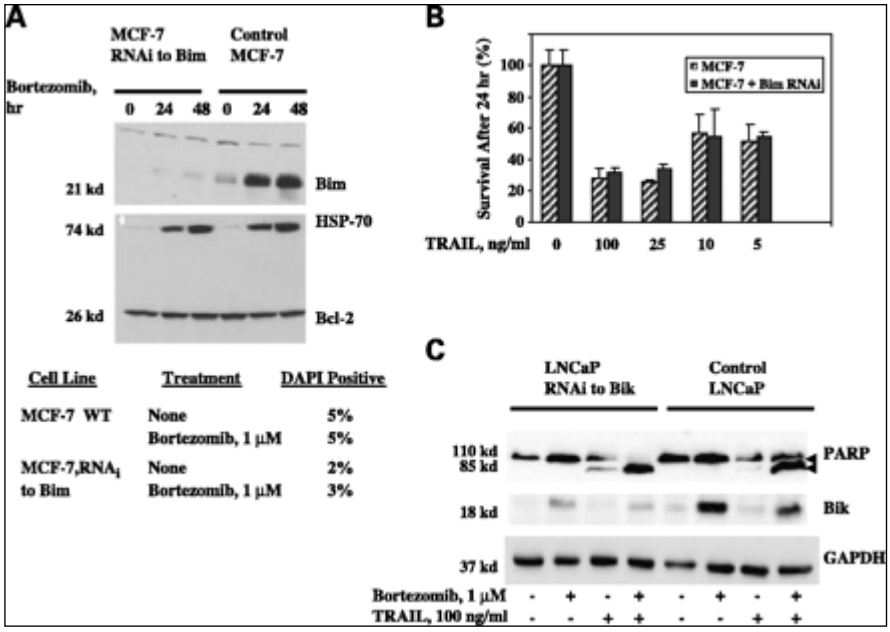

Figure 11. Effectiveness of RNAi directed against Bim and Bik in stable cell lines. **A,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 µmol/L bortezomib for the indicated times. Cell lysates were analyzed by Western blotting for the indicated proteins. *Bottom,* Cells treated with 1 µmol/L bortezomib for 24 h were scored for nuclear DAPI uptake as described in Materials and Methods. **B,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 µmol/L bortezomib and the indicated TRAIL concentrations for 24 h. Surviving cells were scored as described in Materials and Methods. **C,** LNCaP human prostatic cancer cells stably expressing a shRNA against Bik, and the parental cell line, were treated with bortezomib and TRAIL as indicated for 6 h. Cell lysates were analyzed for the indicated proteins by Western blotting.

Similarly, we did not find that knocking out Bik alone increased the cell death induced by PS-341 alone or with the addition of TRAIL. However, we were able to show that the knocking out both Bik and Bim decreased the amount of cell death induced by TRAIL and PS-341.

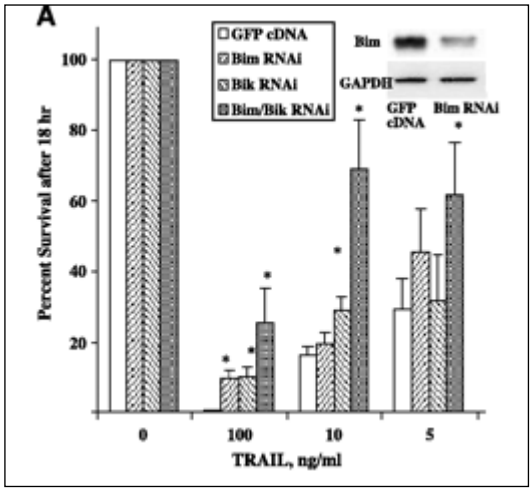

Figure 12. Protection against TRAIL-mediated apoptosis by RNAi directed against Bim and/or Bik. LNCaP human prostatic cancer cells stably expressing a shRNA directed against Bik were transiently transfected with constructs expressing GFP and a shRNA directed against Bim, or the cDNA encoding GFP alone. As a control, LNCaP cells stably expressing an RNAi with no effect on Bik or Bim levels were transiently transfected with constructs expressing the cDNA for GFP and or an shRNA directed against Bim, or the cDNA for GFP alone. Cells expressing GFP were sorted by FACS, plated, and treated with TRAIL at the indicated concentrations in the presence of 1 µmol/L bortezomib for 18 h. Cell survival was scored as described in Materials and Methods. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistically significant deviation from control (GFP) values. *Inset,* Western blot of control LNCaP cells transiently transfected with the constructs expressing GFP and RNAi directed against Bim. GFP-positive and GFP-negative cells were sorted by FACS, lysed, and analyzed for Bim expression by Western blot using GAPDH as a loading control.

These results are exciting because they suggest that both Bim and Bik are essential for death by PS-341. If these proteins are deleted in cancers then they will play a role in making prostate cells insensitive to this drug and thus possibly also to TRAIL.

Unpublished results from our laboratory show that treating cells with PS-341 also increases the protein NOXA. This BH3 protein has been shown to bind to Bak. Thus, we have come full circle. This clearly suggests why Bak is important for the activity of PS-341 and is essential for killing by this agent.

Key Research Accomplishments

- Normal prostate epithelial cells are sensitive to TRAIL-induced apoptosis – *Publication 1*
- Transformation with RAS oncogene sensitizes cells to TRAIL by increasing the amount of caspase 8 bound to FADD – *Publication 4*
- Ras activates Map kinase kinase to activate this caspase 8 binding – *Publication* 4
- Dominate active Map kinase kinase alone will sensitize cells to TRAIL-induced cell death – *Publication 4*
- Prostate cancer cells are uniformly sensitize cells to TRAIL-induced cell death by the proteasome inhibitor PS341 that has now been approved by the FDA for clinical practice *Publication 2*
- TRIAL plus PS-341 kills prostate cancel cells that are Bax negative or overepress Bcl-xL – *Publication 2*
- This drug combination increases cleavage of caspase 8 and Bid secondary in part to increased numbers of TRAIL receptors *Publication 2*
- PS-341 and TRAIL induced killing requires the presence of Bak but not Bax to induce cell death – *Publication 2*
- PS-341 increases the level of BH3 proteins Bim and Bik – *Publication 5*
- RNAi inhibition of Bim and Bik block the ability of TRAIL to kill prostate cancer cells – *Publication 5*
- PS-341 increases the levels of Noxa and MCL-1. (unpublished data) which can bind to Bak.

Reportable Outcomes

1- Nesterov, A., Ivashchenko, Y, and Kraft, A.S. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apopsotosis in normal prostate epithelial cells. Oncogene 21: 1135-1140, 2002.

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Conclusions

We have made a very important observation that TRAIL resistance can be overcome by the addition of the proteasome inhibitor, PS-341, VELCADE. This discovery is important because it suggests that these two agents can be used together to fight cancer. You can treat patients with these agents and have a highly synergistic activity. Clearly in tissue culture these agents can kill prostate cancer. Protocols clearly need to be written to test what they can do in humans.

Additionally, we learn from this research that BH3 proteins can impact on the ability of PS-341 to enhance TRAIL induced death. We also clearly find that PS-341 increases the level of TRAIL receptors. This is essential for the TRAIL induced death regulated by this agent.

This proposal has been successful in generating data that impacts on both the clinical and basic portions of this project.

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells

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TRAIL is a pro-apoptotic cytokine believed to selectively kill cancer cells without harming normal ones. However, we found that in normal human prostate epithelial cells (PrEC) TRAIL is capable of inducing apoptosis as efficiently as in some tumor cell lines. At the same time, TRAIL did not cause apoptosis in several other human primary cell lines: aorta smooth muscle cells, foreskin fibroblasts, and umbilical vein endothelial cells. Compared to these primary cells, PrEC were found to contain significantly fewer TRAIL receptors DcR1 and DcR2 which are not capable of conducting the apoptotic signal. This result suggests that the unusual sensitivity of PrEC to TRAIL may result from their deficiency in anti-apoptotic decoy receptors. The protein synthesis inhibitor cycloheximide significantly enhanced TRAIL toxicity toward PrEC as measured by tetrazolium conversion but had little or no effect on other TRAIL-induced apoptotic responses. Although cycloheximide did not further accelerate the processing of caspases 3 and 8, it significantly enhanced cleavage of the caspase 3 substrate gelsolin, indicating that in PrEC a protein(s) with a short half-life may inhibit the activity of the executioner caspases toward specific substrates. As the majority of prostate cancers are derived from epithelial cells, our data suggest the possibility that TRAIL could be a useful treatment for the early stages of prostate cancer.
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Keywords: TRAIL; apoptosis; primary; prostate; human

TRAIL (TNFα-Related-Apoptosis-Inducing-Ligand)/Apo-2 ligand (Wiley *et al*., 1995; Pitti *et al*., 1996), is a pro-apoptotic cytokine that together with three related proteins, TNFα, CD95/FasL and TWEAK/Apo3L constitute a family of ligands that transduce death signals through death domain containing receptors (Schulze-Osthoff *et al*., 1998). TRAIL has been reported to induce apoptosis in a variety of cancer cells *in vitro*, including colon, breast, lung, kidney, CNS, blood and skin (Wiley *et al*., 1995; Ashkenazi *et al*., 1999; Walczak *et al*., 1999, 2000; Griffith *et al*., 1999), as well as in colon and breast tumor implants in nude mice (Ashkenazi *et al*., 1999, Walczak *et al*., 1999). The chemotherapeutic agents 5-FU and CPT-11 can further enhance the cytotoxic effects of TRAIL (Ashkenazi *et al*., 1999). At the same time, a large panel of primary human cells including prostate epithelial cells has been tested and reported to be TRAIL resistant (Ashkenazi *et al*., 1999; Walczak *et al*., 1999). Moreover, unlike TNFα and Fas ligand, whose use for cancer therapy had been hampered by their severe toxicity *in vivo* (Vassalli, 1992; Nagata, 1997), TRAIL had no toxic effects when systemically administered in rodents (Walczak *et al*., 1999) and non-human primates (Ashkenazi *et al*., 1999). These experimental data lead to the general belief that TRAIL can be used as a safe and specific anti-cancer agent.

Previously, we investigated the effects of TRAIL on several prostate cancer cell lines and found that their responses to TRAIL ranged from being highly sensitive (ALVA-31, DU-145, PC-3) to partially (JCA-1, TSU-Pr1) or completely (LNCaP) resistant (Nesterov *et al*., 2001). Because TRAIL is a potential treatment for human prostate cancer, we next investigated how normal human prostate cells respond to this cytokine.

Incubation of normal human prostate epithelial cells (PrEC) with TRAIL for 6 h induced morphological changes that are characteristic of apoptosis (Figure 1a), including cell rounding and shrinkage, nuclear condensation, and membrane blebbing. To confirm that the observed changes constitute apoptosis, we tested whether TRAIL induced DNA fragmentation, a hallmark of apoptotic cell death. Figure 1b demonstrates that treatment of PrEC for 6 h with 100 ng/ml of TRAIL induced significant DNA fragmentation. To quantitate TRAIL-induced DNA fragmentation, we employed an assay that measures the release of free nucleosomes into the cytosol of apoptotic cells. Figure 1c demonstrates that TRAIL-induced DNA fragmentation reaches a maximal level at a TRAIL concentration of 60 ng/ml. Comparing these data with the published results on the sensitivity of various cancer cell lines to TRAIL-induced apoptosis (Ashkenazi *et al*., 1999; Walczak *et al*., 1999, 2000; Griffith *et al*., 1999), PrEC could be classified as highly

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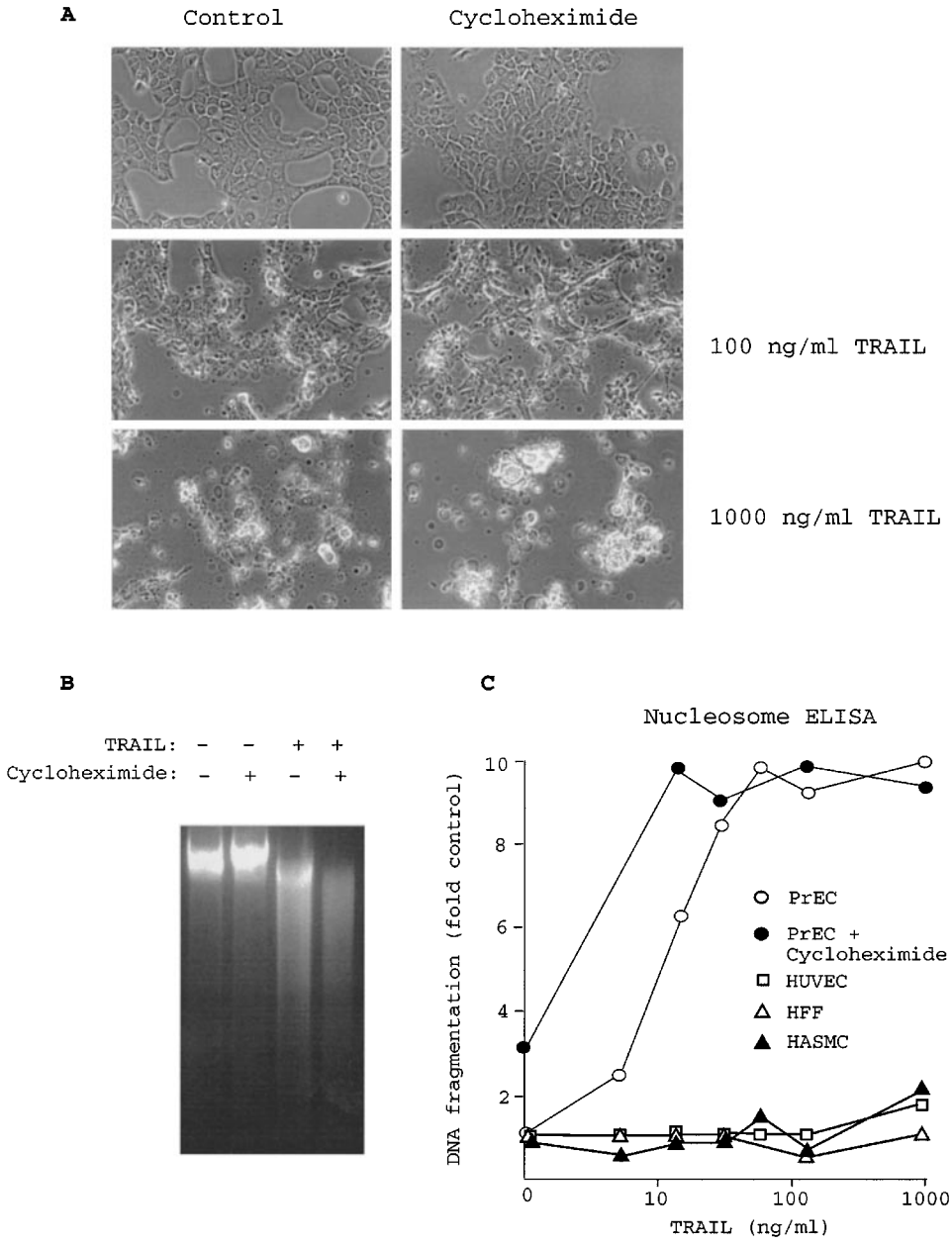

Figure 1 (**a**) Normal prostate epithelial cells (PrEC) were pre-treated overnight with 10 μM cycloheximide or left untreated (Control). Cultures were then incubated for 2 h with 100 or 1000 ng/ml of TRAIL, and visualized by light microscopy. (**b**) PrEC were pre-treated with 10 μM cycloheximide overnight or left untreated. Where indicated, cells were then incubated with 100 ng/ml of TRAIL for 6 h and DNA fragmentation was monitored by electrophoresis in a 2% agarose gel. (**c**) PrEC, human foreskin fibroblasts (HFF), human umbilical vein endothelial cells (HUVEC), and human aorta smooth muscle cells (HASMC) were incubated for 6 h with TRAIL at concentrations increasing from 7–1000 ng/ml. DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone complexes released into cytoplasm using Cell Death Detection ELISA^{plus} Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). In one case PrEC were pre-treated overnight with 10 μM cycloheximide prior to the experiment. All primary huamn cells were purchased from Clonetics (Biowhittaker Products, Walkersville, MD, USA) and maintained as outlined by the supplier. The expression and purification of recombinant TRAIL was described in detail elsewhere (Nesterov *et al*., 2001). Briefly, soluble TRAIL was generated by expression of amino acids 114–281 of human TRAIL fused with amino-terminal $(His)_6$ tag in yeast *Pichia pastoris*. The secreted protein was purified to homogeneity from the yeast supernatant by Ni-chelate chromatography

TRAIL-sensitive. Since this finding contradicted the general concept that TRAIL toxicity is limited to cancerous cells, we tested whether our preparation of TRAIL possessed an aberrant toxicity toward normal cells. It was found that our TRAIL protein did not cause apoptosis in several other primary cells: human foreskin fibroblasts (HFF), human umbilical cord endothelial cells (HUVEC), and human aorta smooth muscle cells (HASMC) (Figure 1c). Consistent with previously published data (Ashkenazi *et al*., 1999; Walczak *et al*.,

1999), systemic administration of our TRAIL to athymic mice for up to 10 days (100–500 μg/animal) did not produce any toxic effects (data not shown). Compared with two prostate cancer cell lines, ALVA-31 and LNCaP cells, PrEC displayed an intermediate sensitivity to TRAIL (Figure 2a,b). Thus, contrary to the concept that malignant cells are more sensitive to this protein than normal ones (Wiley *et al*., 1995; Leverkus *et al*., 2000), it appears that cells may either decrease or increase their sensitivity to this agent upon transformation.

The finding that, unlike some other human primary cells, PrEC appear to be unusually sensitive to TRAIL raised the question of whether these cells contain an abnormally high number of TRAIL receptors. Quantitative PCR data (Figure 2c) demonstrated that the amount of mRNA for TRAIL receptors DR4 and DR5 in PrEC was comparable to that found in TRAIL-resistant HUVEC and HASMC. However, the proportional amount of mRNA for TRAIL receptors DcR1 and DcR2, which bind TRAIL but do not transduce a death signal, was significantly lower in PrEC and TRAIL-sensitive prostate cancer cells ALVA-31 than in other primary cells and TRAIL-resistant prostate cancer cells LNCap. Thus, one possible explanation for the unusual sensitivity of PrEC to TRAIL is that these cells have fewer 'decoy' receptors.

Since our data contradicted the previous report (Ashkenazi *et al*., 1999) where PrEC were found to be TRAIL-resistant, we performed a series of experiments to confirm the specificity of our observation. First, to

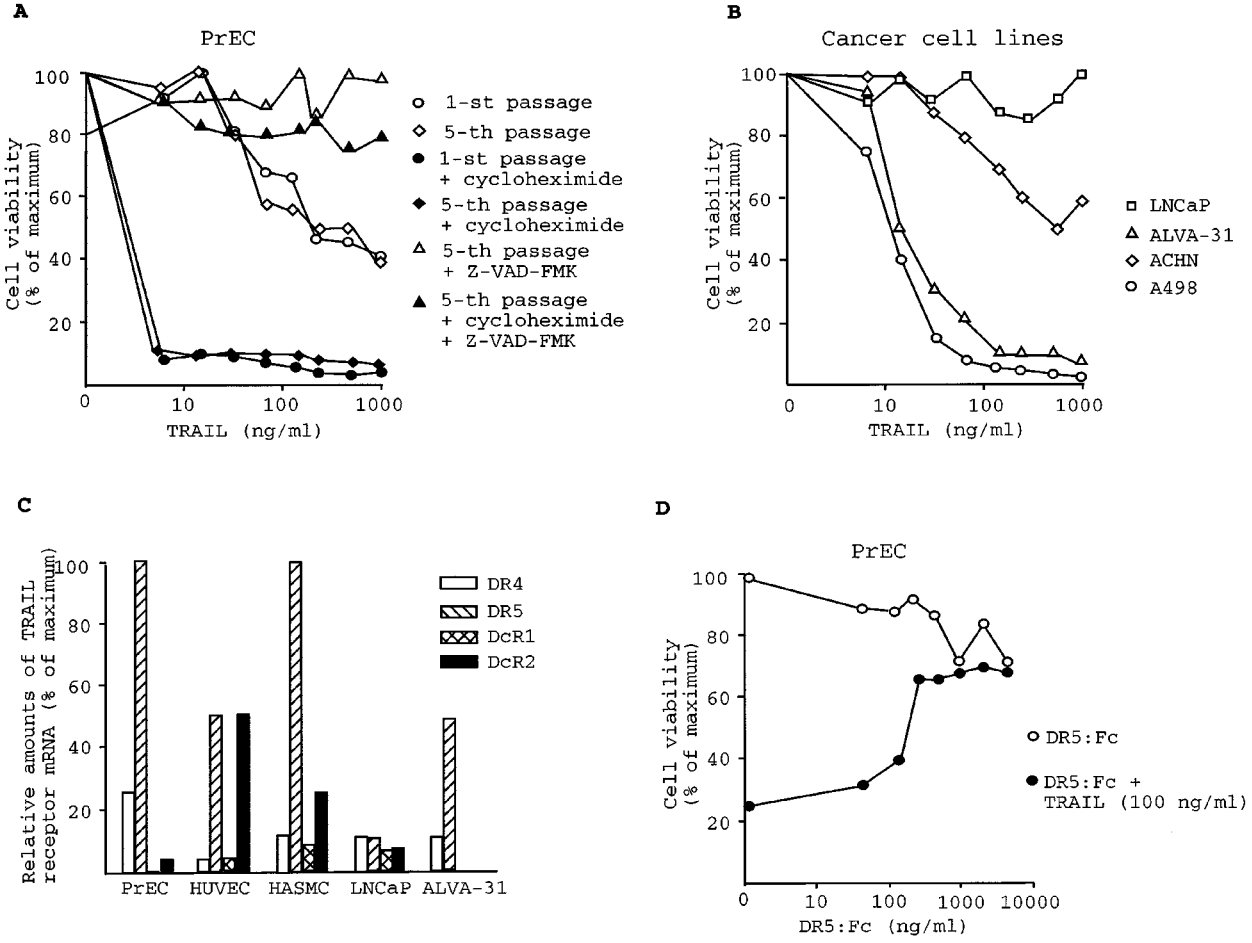

Figure 2 (**a**) Normal human prostate epithelial cells on 1st and 5th passages were pre-treated with 10 μM cycloheximide overnight or left untreated. Cells were then incubated for 24 h with TRAIL alone at concentrations increasing from 7–1000 ng/ml or in combination with 40 μM of Z-VAD-FMK (Enzyme Systems Products, Livermore, CA, USA). Cell viability was determined spectrophotometrically using an MTS tetrazolium based assay (Promega, Madison, WI, USA). Absorbance was measured at 490 nm and data from quadruplicate determinations was plotted as per cent of maximal signal. (**b**) Human prostate cancer cell lines LNCaP and ALVA-31, and renal cancer cell lines ACHN and A498, were incubated for 24 h with TRAIL at concentrations increasing from 7–1000 ng/ml. Cell viability was determined as described in the legend to **a**. (**c**) Relative amounts of mRNA for pro-apoptotic TRAIL receptors DR4 and DR5 and 'decoy' receptors DcR1 and DcR2 in primary cells and prostate cancer cell lines were determined by quantitative PCR using β-actin as a standard. RNA was extracted from cells using TRIzol reagent (GibcoBRL, Rockville, MD, USA). Real time PCR was performed using iCycler (BioRad, Hercules, CA, USA). (**d**) PrEC were treated for 36 h with soluble extracellular domain of TRAIL receptor DR5 (DR5:Fc) (Alexis, San Diego, CA, USA) alone at concentrations increasing from 64 to 8000 ng/ml or in the presence of 100 ng/ml of TRAIL. Cell viability was determined as described in the legend to **a**

rule out the possibility that TRAIL produced in our laboratory is significantly more potent than that used by others, we tested its efficacy on two renal cancer cell lines, ACHN and A498 (Figure 2b), which were also examined by Ashkenazi *et al*., 1999. The results we obtained using our TRAIL preparation were comparable to the published data: 100 ng/ml of TRAIL reduced cell viability approximately 2–5-fold for ACHN and A498 cells respectively. Second, using pan-caspase inhibitor Z-VAD-FMK, we confirmed that the effect of our TRAIL on PrEC was mediated by caspases (Figure 2a), suggesting that our TRAIL was activating the normal apoptotic pathway. Third, we found that the soluble extracellular domain of TRAIL receptor DR5 (DR5:Fc) is capable of inhibiting TRAIL-induced apoptosis in PrEC (Figure 2d), indicating that the effect of TRAIL is receptor-mediated and does not result from a toxic contaminant of our preparation.

Since the response of cells to various apoptotic stimuli may change with aging (Warner *et al*., 1997) we also tested whether the conflicting results could arise from the differences in the age of cell cultures. In our experiments PrEC did not change their growth characteristics during the first five passages, with senescent cells beginning to appear after passage six. The data presented in Figure 2a demonstrate that young (1st passage) and aged (5th passage) cultures are equally sensitive to TRAIL.

It is possible that the discrepancies between our results and those previously published may arise from the assays chosen to quantitate apoptosis. For example, in the apoptosis-specific DNA fragmentation assay, internucleosomal DNA fragmentation reached a maximum at a TRAIL concentration of 60 ng/ml (Figure 1c). However, in the tetrazolium conversion assay, a technique that measures general cell viability (Cory *et al*., 1991), the maximal effect could not be achieved even at 1000 ng/ml of TRAIL (Figure 2a). In the report by Ashkenazi *et al*., 1999, PrEC were found to be TRAIL-insensitive, judging by propidium iodide staining. A possible drawback to the use of vital dyes, such as propidium iodide, is that cells undergoing apoptosis may retain cell membrane integrity and appear alive until late in the apoptotic program when secondary necrosis begins (Loo and Rillema, 1998). For example, in our experiments, staining of PrEC with vital dye, trypan blue, did not detect a significant number of dead cells if they were treated with TRAIL for less than 24 h (data not shown).

The pro-apoptotic effects of TRAIL can often be enhanced by the inhibition of protein synthesis (Griffith *et al*., 1998; Kreuz *et al*., 2001; Wajant *et al*., 2000), suggesting that proteins with a short half-life protect cells from TRAIL-induced death. Therefore, we tested whether the inhibition of protein synthesis by cycloheximide affects TRAIL-induced apoptosis in PrEC. As demonstrated by Figure 1, cycloheximide had little or no effect on TRAIL-induced morphological changes and only moderately increased sensitivity of cells to this agent in DNA fragmentation assays. However, as measured by the tetrazolium conversion assay (Figure 2a) which assesses cell viability based on the respiratory function of mitochondria (Cory *et al*., 1991), the inhibition of protein synthesis dramatically enhanced TRAIL toxicity. These results suggest that some, but not all TRAIL-induced apoptotic responses in PrEC are partially inhibited by short-lived anti-apoptotic proteins. As demonstrated by Figure 3, processing of the initiator caspase 8, the effector caspase 3, and one of caspase 3 substrates, the inhibitory subunit of DNA fragmentation factor (DFF45/ICAD) (Cryns and Yuan, 1998), were induced by TRAIL alone as efficiently as when TRAIL was combined with cycloheximide. In contrast, cyclohex-

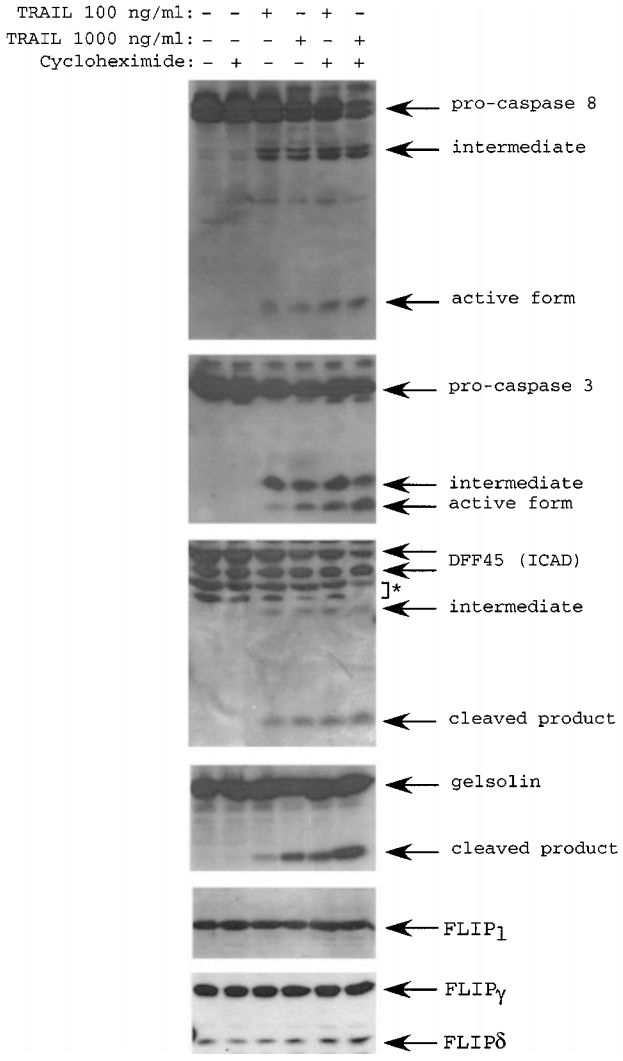

Figure 3 PrEC were pre-treated overnight with 10 μM cycloheximide or left untreated. Cultures were then incubated for 6 h with 100 or 1000 ng/ml of TRAIL alone or in combination with cycloheximide. Cell lysates were electrophoresed and the same Western blot was consecutively probed with antibodies specific to caspase 8 (Upstate Biotechnology, Lake Placid, NY, USA), caspase 3 (Transduction Laboratories, Lexington, KY, USA), DFF45 (Zymed Laboratories, So. San Francisco, CA, USA), gelsolin (Sigma, St. Louis, MO, USA), FLIP$_l$ (ABR, Golden, CO, USA) and FLIP γ/δ (Calbiochem, San Diego, CA, USA)

imide significantly enhanced processing of gelsolin, a prominent caspase 3 substrate, implicated in mediating apoptotic cytoskeletal changes (Kothakota *et al*., 1997). This result indicates that in PrEC a short-lived anti-apoptotic proteins(s) may inhibit the activity of the effector caspases toward specific substrates.

It has been recently reported that the inhibition of protein synthesis significantly enhances TRAIL-induced apoptosis in primary human keratinocytes (Kothakota *et al*., 1997). In keratinocytes, cycloheximide down-regulated FLIPs, proteins implicated in caspase 8 inhibition (Tschopp *et al*., 1998) and accelerated TRAIL-induced caspase 8 processing. However, in PrEC cycloheximide did not affect either the level of FLIPs or the rate of caspase 8 processing (Figure 3). This result indicates that the biologic effects of cycloheximide may be cell type specific.

Together with the recent data on TRAIL cytotoxicity toward primary human keratinocytes (Leverkus *et al*., 2000) and hepatocytes (Jo *et al*., 2000) our results challenge the concept of the cytotoxic effects of TRAIL being limited to transformed cells. While this paper was under review, Lawrence *et al*., 2001 reported that different preparations of TRAIL may have different effects on primary cells. Based on the observation that recombinant TRAIL containing a hexahistidine tag induced apoptosis in primary human hepatocytes, whereas TRAIL without this tag did not, the authors concluded that the toxicity of TRAIL toward normal cells depended on the presence of hexahistidine. However, the data on primary human keratinocytes demonstrated that recombinant TRAIL which lacks a hexahistidine sequence may also trigger apoptosis in certain normal cells (Lawrence *et al*., 2001). Since we were not able to obtain TRAIL preparations used in the above referenced studies, we cannot preclude that the toxicity of our TRAIL toward prostate epithelial cells resulted from the presence of a hexahistidine tag. However, the effect of our TRAIL on PrEC did not appear to result from non-specific toxicity because: (1) it was dependent on caspase activity; (2) it could be inhibited by the extracellular domain of TRAIL receptor; and (3) it was accompanied by proteolytic events typical of receptor-mediated apoptosis. The observation that PrEC were unusually sensitive to TRAIL may have important implications for TRAIL-based prostate cancer therapy. As the majority of prostate cancers are derived from the epithelial cells (Stamey and McNeal, 1992), it is possible that TRAIL could be used for the treatment of developing premalignant lesions or early stages of prostate cancer. At the same time, our data raise concerns about TRAIL safety when this agent is used as a systemic drug for cancer therapy. It cannot be ruled out that some other human tissues may also be sensitive to this agent; thus, more extensive studies are needed to evaluate TRAIL sensitivity of multiple other primary cell lines before TRAIL is used for human treatment. A better understanding of the mechanisms involved in the inhibition of receptor-mediated cell death may also be beneficial for deciphering control of TRAIL-induced apoptosis in normal and malignant cells.

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The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells

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We demonstrate that PS-341, a small molecule inhibitor of the proteasome, markedly sensitizes resistant prostate, colon, and bladder cancer cells to TNF-like apoptosis-inducing ligand (TRAIL)-induced apoptosis irrespective of Bcl-xL overexpression. PS-341 treatment by itself does not affect the levels of Bax, Bak, caspases 3 and 8, c-Flip or FADD, but elevates levels of TRAIL receptors DR4 and DR5. This increase in receptor protein levels is associated with the ubiquitination of the DR5 protein. When PS-341 is combined with TRAIL, the levels of activated caspase 8 and cleaved Bid are substantially increased. In Bax-negative TRAIL-resistant HC-4 colon cancer cells, the combination of PS-341 and TRAIL overcomes the block to activation of the mitochondrial pathway and causes SMAC and cytochrome *c* release followed by apoptosis. Similarly, murine embryonic fibroblasts lacking Bax undergo apoptosis when exposed to the combination of PS-341 and TRAIL; however, fibroblasts lacking Bak are significantly resistant. Taken together, these findings indicate that PS-341 enhances TRAIL-induced apoptosis by increasing the cleavage of caspase 8, causing Bak-dependent release of mitochondrial proapoptotic proteins.
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Keywords: PS-341; TRAIL; Bax; Bcl-2; apoptosis

Introduction

TNF-like apoptosis-inducing ligand (TRAIL) and PS-341 are two novel anticancer agents with differing mechanisms of action. TRAIL induces apoptosis by first binding to two closely related receptors DR4 and DR5, causing the formation of a death-inducing signaling complex (DISC), which includes the receptors, the adaptor protein FADD, and caspase 8 (Medema *et al*., 1997). Autoactivated caspase 8 is capable of cleaving procaspase 3 directly or digesting Bid to its active form (tBid), which leads to the release of cytochrome *c* from the mitochondria (Li *et al*., 1998; Luo *et al*., 1998; Gross *et al*., 1999). PS-341 is a small molecule inhibitor of the proteasome that induces apoptotic cell death (Teicher *et al*., 1999). Although treatment with this compound causes marked increases in a large number of cellular proteins (Adams *et al*., 1999), including c-*myc* and the cyclin-dependent protein kinase inhibitor $p21^{WAF1}$, it is not clear how this agent actually induces apoptosis. Tumor cells that do not express p53 or p21 still die when incubated with this agent (An *et al*., 2000). PS-341 inhibits the activation of NF-κB (Sunwoo *et al*., 2001). NF-κB plays a role in controlling the levels of inhibitor of apoptosis proteins, IAPs (Wang *et al*., 1998); however, the regulation of NF-κB activity has not been directly linked to a specific mechanism by which PS-341 induces apoptosis. Through an unknown mechanism, PS-341 treatment of tumor cells overcomes drug resistance, and can enhance the killing activity of both dexamethasone and chemotherapeutic agents (Cusack *et al*., 2001; Hideshima *et al*., 2001). This effect would appear additive and not synergistic.

Despite widespread expression of TRAIL receptors, many tumor cell lines are either partially or completely resistant to apoptotic cell death induced by this agent (Hao *et al*., 2001; Pawlowski *et al*., 2001). Resistance results from a number of factors including the absence of caspase 8 (Grotzer *et al*., 2000), increased AKT activity resulting from deletion of the PTEN phosphatase (Nesterov *et al*., 2001), or the absence of Bax (Deng *et al*., 2002). In addition, overexpression of Bcl-2 family members blocks TRAIL-induced apoptosis (Munshi *et al*., 2001; Roklin *et al*., 2001). The observation that the majority of TRAIL-resistant cell lines can be made TRAIL sensitive by treatment with protein synthesis inhibitors (Rieger *et al*., 1998; Wajant *et al*., 2000) implies that additional mechanisms regulating TRAIL resistance may exist.

Our results indicate that PS-341 and TRAIL synergize to kill TRAIL-insensitive tumor cell lines, including those overexpressing Bcl-xL or deficient in Bax, but is much less effective in cells lacking Bak. The effects of PS-341 appear to be mediated by its ability to enhance TRAIL-stimulated cleavage of caspase 8, which may be correlated with increased expression of TRAIL recep-

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tors DR4 and DR5. Our results suggest that this combination of agents may be useful therapeutically in killing tumor cells with multiple abnormalities in the apoptotic pathway.

Materials and methods

Cell lines and reagents

The human prostatic carcinoma cell line LNCaP and bladder JCA-1 cells were cultured in RPMI 1640 (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, 100 U/ml penicillin/100 μg/ml streptomycin. HCT-116, HC-4, and murine embryonic fibroblasts (MEF) were cultured in DMEM (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, 100 U/ml penicillin/100 μg/ml streptomycin. MEF were frozen at passage 2 and used at the first passage after thawing. Antibodies were obtained from the following sources: DR4 and DR5 antibodies, Alexis (San Diego, CA, USA); ubiquitin antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Bcl-2, caspase 8, Upstate Biotechnology (Lake Placid, NY, USA); Poly ADP ribose polymerase (PARP), Trevigen (Gaithersburg, MD, USA); $P21^{waf1}$ and FADD antibodies, BD Transduction Laboratories (San Jose, CA, USA); SMAC and myc antibodies, University of Colorado Health Science Center Monoclonal Antibody Core Facility (Denver, CO, USA); FLAG agarose, FLAG and HA antibodies, Sigma Chemical Co. PS-341 was a gift of Millennium, Inc. (Cambridge, MA, USA). The FLAG-DR5 L334N expression vector was constructed as follows: the sequence corresponding to residues 56–411 of mature human DR5 was amplified from a template supplied by Genentech. This sequence was inserted into the expression vector pFLAG-CMV1 (Sigma) to provide an N-terminal signal peptide from preprotrypsin and the FLAG epitope. The L334N mutation in the death domain was then made with the QuickChange kit (Stratagene), and the entire epitope-bearing sequence transferred to the episomal vector pCEP4 (Invitrogen). The integrity of the construct was verified by DNA sequencing.

Recombinant human TRAIL

TRAIL cDNA was subcloned into the pET-15b vector (Novagen, Madison, WI, USA) and used to transform *Escherichia coli* strain BL21 (Promega, Madison, WI, USA). Recombinant human TRAIL was purified on a Ni-NTA agarose column (Qiagen, Valencia, CA, USA) after cell lysis in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 1 mM PMSF, 5 mM 2-mercaptoethanol, and 2.5% Triton X-100.

Cell viability assays

To measure cell viability, LNCaP and LNCaP-Bcl-xL cells were plated in 96-well plates at 10 000 cells/well and incubated overnight. The cells were then treated with varying amounts of DMSO, PS-341, TRAIL, or the combination. After a 16 h incubation at 37°C, MTS substrates (Cell TITER 96 Aqueous Assay, Promega, Madison, WI, USA) were added according to protocol, and absorbance at 490 nm was measured with a *v*Max Kinetic Plate Reader (Molecular Devices, Sunnyvale, CA, USA). For assays employing 4′,6-diamido-2-phenylindole (DAPI), cells were plated in six-well plates 24 h prior to treatments. At the end of the treatment period, cells were washed, trypsinized, centrifuged, and resuspended in 0.05 ml complete medium containing 1 μg/ml DAPI. All components of the harvesting step (culture medium, washes, and trypsinized cells) were combined before centrifugation to ensure recovery of unattached cells. Cells were examined and nuclei with apoptotic morphology were counted using a Zeiss Axiophot fluorescence microscope with an appropriate filter at $\times 400$ magnification. DAPI experiments were performed in duplicate and a minimum of 300 cells per sample were scored.

Western blot analysis

Cell lines were plated in six-well plates and treated with appropriate amounts of DMSO as a control, PS-341, or TRAIL under a variety of conditions. The cells were then lysed in SDS–PAGE sample buffer (15% glycerol, 0.125 M Tris-HCl, pH 6.8, 5 mM EDTA, 2% SDS, 0.1% bromophenol blue, 1% 2-mercaptoethanol), and incubated for 10 min at room temperature. The lysate was then boiled for 5 min and frozen at -80°C. Western blots were carried out as described previously (Nesterov *et al*., 2001).

Quantitative RT–PCR

Quantitative RT–PCR assays were performed using the ABI 5700 real-time system with SYBR-green fluorescence, as described in detail (Drabkin *et al*., 2002). Briefly, cDNA was synthesized from 2.5 μg of total RNA isolated by the method of Chomczynski and Sacchi (1987) with Superscript II (Invitrogen Life Technologies, Carlsbad, CA, USA) using random primers and conditions recommended by the manufacturer. For quantitative RT–PCR, 20 μl reactions were utilized, which included 1 μl cDNA, 1.6 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphates, 0.1 μM primers, and 0.1 μl AmpliTaq Gold (Applied Biosystems, Wellesley, MA, USA). Following an initial 95°C 10 min incubation to activate the polymerase, 40 cycles of a two-stage PCR were performed consisting of 95°C $\times$15 s and 60°C $\times$1 min. Duplicate reactions were performed that invariably agreed within 1/2 cycle. RNA used for cDNA synthesis was measured both by optical density and gel electrophoresis followed by densitometric scanning. PCR products were verified by gel analysis. Absence of contamination was verified by the use of no template controls and mock cDNA synthesis reactions without reverse transcriptase. Data were analysed with GeneAmp 5700 SDS software. Primers were DR4F: ATGGCGCCACCACCAGCTAGAG; DR4R: GGAGGTAGGGAGCGCTCCTCGG; DR5F: ATCACCCAACAAGACCTAGC; DR5R: CACCTGGTGCAGCGCAAGCAG. Semiquantitative PCR for DR5 was performed with cDNAs prepared as above. Primers were DR5F: GGGAAGAAGATTCTCCTGAGATGT; DR5R: ACATTGTCCTCAGCCCCAGGTCG, which span the splice junction (Screaton *et al*., 1997).

Isolation of TRAIL receptors DR4 and DR5

Cells were detached from plates by incubation with Hank's Basic Salt Solution supplemented with 2 mM EDTA and 2 mM EGTA. The isolation was performed in two ways. (1) For isolation of total cellular receptors, cells were pelleted, washed in PBS, and lysed in TGH buffer (50 mM HEPES pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.25 mM phenylmethylsulfonylfluoride, and protease inhibitors) for 15 min on ice. Lysates were centrifuged for 30 min at 100 000 *g*. Histidine-tagged TRAIL prepared as described above was added at 1 μg/ml to the supernatants, which were incubated for 20 min on ice. Anti-histidine antibody coupled to agarose beads (Sigma) was added to the supernatants, which were then incubated with rotation for 2 h to overnight at 4°C. Beads were washed

thoroughly with TGH buffer and eluted twice with 100 mM glycine (pH 2.3) for 10 min on ice. Following addition of concentrated SDS–PAGE sample buffer, the eluates were neutralized with 1 N NaOH and boiled for 5 min prior to electrophoresis. (2) For isolation of cell surface receptors and associated DISC components, following detachment from culture dishes, cells were pelleted and resuspended in culture medium set aside from the individual treatments. Histidine-tagged TRAIL was added to 1 μg/ml and the cells were incubated at 37° for 20 min with agitation. After assessment of viability by trypan blue exclusion, cells were pelleted, washed, lysed, and centrifuged as described above. The remainder of the procedure was as described except that TRAIL was not added to the cell lysates. Before the addition of anti-histidine agarose, the protein content of the lysates was determined and volumes adjusted to equalize protein content.

Cytosolic SMAC levels

Cytosolic extracts from HC-4, HCT-116, and LNCaP cells were prepared by the procedure originally described by Bossy-Wetzel *et al*. (1998) as modified by Nesterov *et al*. (2001). Briefly, 10-cm plates of cells near-confluence were scraped directly into cell culture medium and pelleted for 5 min at 200 *g*. Cells were washed once in 1 ml phosphate-buffered saline (PBS) and resuspended in 0.35 ml HT buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES–KOH pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) by vortexing. After 45 min on ice, cells were revortexed and passed 10 times through a 25 gauge needle. Following 10 min centrifugation at 200 *g*, the supernatant was cleared by centrifugation for 30 min at 100 000 *g* and analysed for SMAC protein by Western blotting.

Nucleosome assay

LNCaP cells were grown to confluency in 10-cm plates. They were then treated with DMSO, 1 μM PS-341, 100 ng/ml TRAIL, or a combination of PS-341 and TRAIL. The following day, the cells were collected via trypsinization, pelleted and lysed according to the Nucleosome ELISA kit (Oncogene Research Products, Boston, MA, USA). The subsequent assay was carried out as directed.

Crystal violet staining

HCT-116 and HC-4 cells were plated in 24-well plates and grown to confluency. They were then treated with varying concentrations of PS-341 and/or TRAIL. Medium was gently aspirated and cells were washed in 1 × PBS. Crystal violet stain was prepared with 0.1% crystal violet in ddH_2O and added to wells until stain covered the bottom of the wells. The plates were then incubated at room temperature for 10 min. The stain was then gently aspirated and the wells were washed three times with 1 × PBS.

X-Gal staining of caspase 8-transfected cells

HCT-116 and HC-4 cells were plated in six-well plates and grown to approximately 50% confluency. They were then cotransfected with 0.2 μg LacZ plasmid (Invitrogen) and 0.2 μg Fkp-caspase 8 (180) plasmid (a gift of Dr David Baltimore, California Institute of Technology, Pasadena, CA, USA). The cells were incubated for 24 h and stained with X-Gal. The experiment was performed in triplicate and repeated twice.

Results

To determine whether PS-341 enhances the ability of TRAIL to induce cell death, LNCaP human prostate cancer cells were incubated with either TRAIL, PS-341, or the combination for 24 h and a cell viability (MTS) assay was carried out. LNCaP cells showed no change in viability after treatment with either PS-341 or TRAIL, but the combination of both led to significant decrease in viability (Figure 1a). Using the analysis of Chou and Talalay (1983), the combination of these two agents was found to be highly synergistic (data not shown). To establish that the decrease in viability was caused by induction of programmed cell death (apoptosis), an ELISA assay was performed, which measures free cleaved nucleosomes generated during apoptosis. Only addition of both PS-341 and TRAIL, but not TRAIL or PS-341 alone, induced increases in free nucleosomes (Figure 1b). Similar results were obtained with multiple prostate cancer cell lines, including PC-3 and DU-145 cells (data not shown).

To evaluate the possibility that the failure of PS-341 alone to kill LNCaP cells reflected an inability of the drug to penetrate these cells, levels of specific cellular proteins after treatment were measured. Since PS-341 inhibits proteasome degradation, we expected to see an increase in ubiquinated proteins. An 18 h treatment of LNCaP cells with PS-341 caused marked increases in total ubiquinated proteins, as well as two proteins normally degraded by the proteasome system, $p21^{WAF-1}$ and c-*myc* (Figure 1c), whereas there was no change in the levels of PARP protein. It is possible that the increase in $p21^{WAF-1}$ seen when incubation with PS-341 is compared to the combined treatment may come from an increased ability to extract this protein in cells undergoing apoptosis. Only the combination of PS-341 plus TRAIL, but not PS-341 alone, induced the cleavage of PARP (Figure 1c), consistent with the ability of these agents when combined to induce apoptosis.

Overexpression of Bcl-2 family members blocks TRAIL-induced cell death (Munshi *et al*., 2001; Roklin *et al*., 2001), and release of SMAC and cytochrome *c* from mitochondria. To test whether PS-341 could overcome this block, LNCaP prostate cancer cells overexpressing the Bcl-xL protein were incubated with PS-341, TRAIL, or the combination (Figure 2a). Approximately threefold overexpression of Bcl-xL compared to parental LNCaP cells was verified (data not shown). MTS assay results demonstrated that whereas each agent alone was unable to decrease cell viability, the combination was able to overcome Bcl-xL overexpression, leading to marked decrease in cell viability. Parallel experiments demonstrated that only the combination of PS-341 and TRAIL resulted in the appearance of SMAC protein in cytosolic extracts of these cells (data not shown).

TRAIL-induced cleavage of Bid to tBid enables the translocation of Bax protein into mitochondria followed by the release of cytochrome *c* and induction of apoptosis (Wei *et al*., 2001). To investigate whether TRAIL and PS-341 could induce the death of cells that

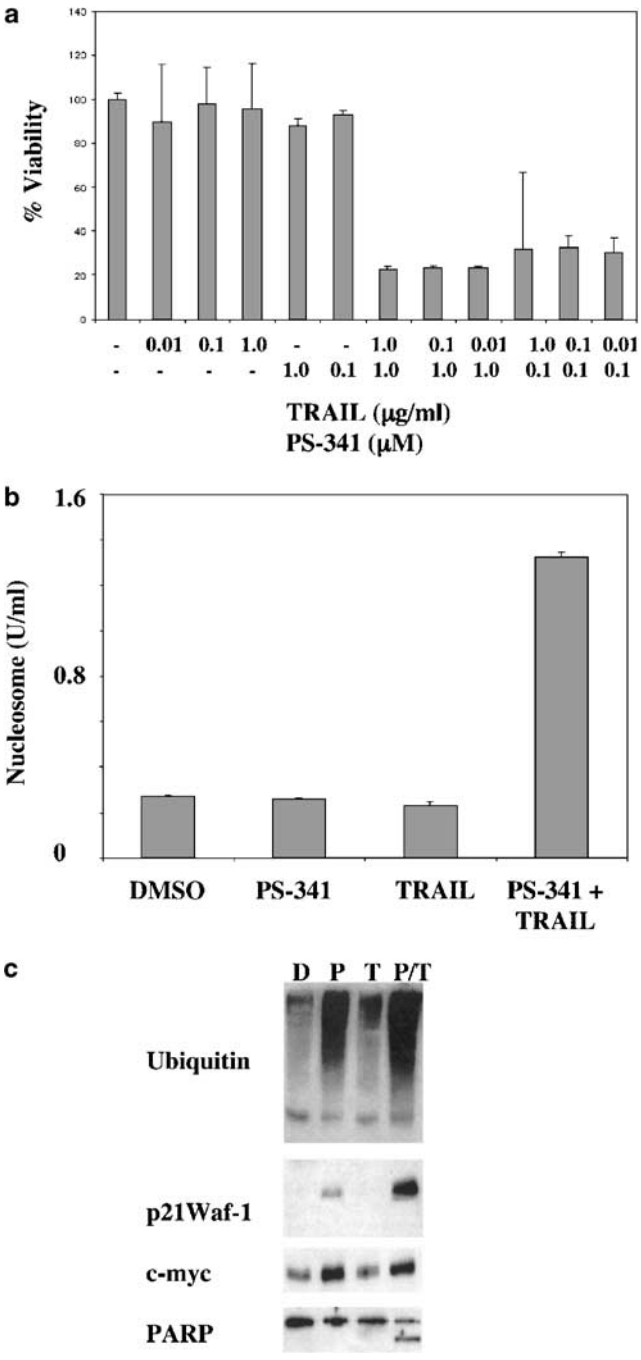

Figure 1 PS-341 treatment markedly enhances TRAIL-induced apoptosis. (**a**) Cell viability assay of cell growth. LNCaP cells were treated with PS-341 or TRAIL in varying amounts overnight and the MTS assay was performed as described in Materials and methods. The s.d. of triplicate determinations is shown. (**b**) Nucleosome ELISA assay of treated LNCaP cells. LNCaP cells were treated overnight with vehicle, PS-341 (1 μM), TRAIL (0.1 μg/ml), or the combination of these two agents. The s.d. of triplicate determinations is shown. (**c**) The effect of PS-341, TRAIL, or both agents on the levels of specific cellular proteins. LNCaP cells were treated as in Figure 1b (D, DMSO; T, TRAIL; P, PS-341, and P/T, PS-341 and TRAIL). Extracts were run on a 10% SDS : PAGE gel, transferred to Immobilon, and Western blotted with antibodies to ubiquitin, p21^{waf-1}, c-Myc, and PARP

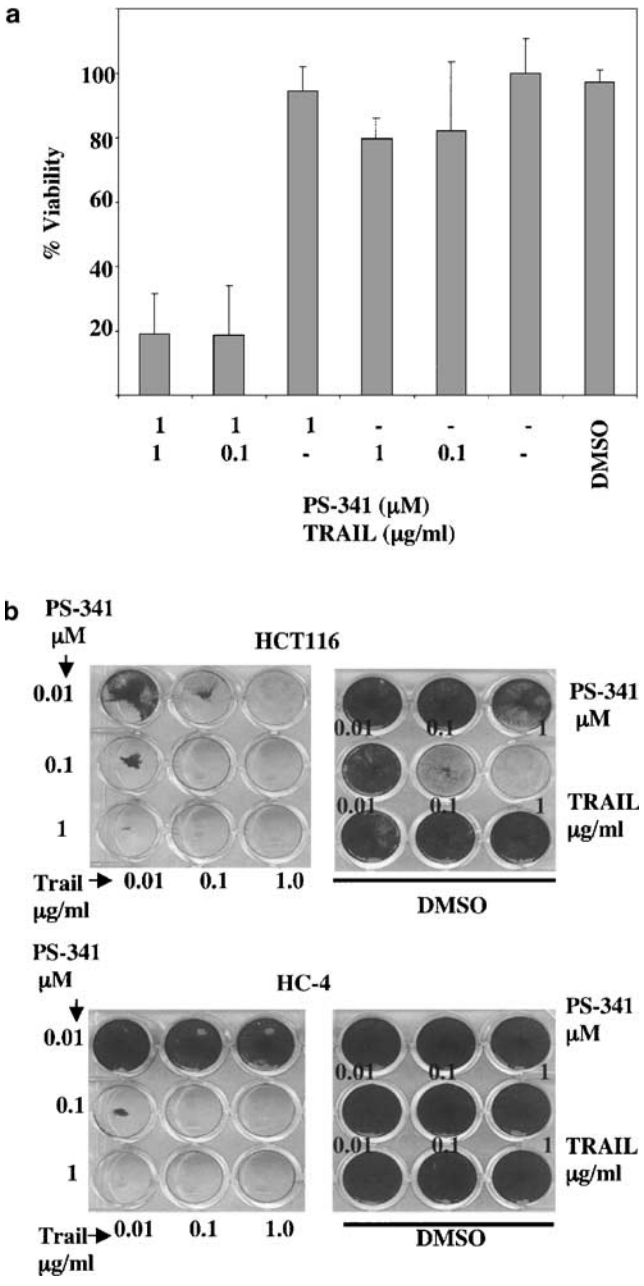

Figure 2 The combination of TRAIL and PS-341 overcomes Bcl-xL overexpression and the absence of Bax to induce cell death. (**a**) LNCaP-Bcl-xL cells were treated overnight with either PS-341 or TRAIL or both agents. An MTS assay was performed. The s.d. of measurements made in triplicate is shown. (**b**) Treatment of HCT-116 and HC-4 cells with varying concentrations of PS-341 (P) or TRAIL (T) or both agents. Cells were treated overnight and then stained with crystal violet as described in Materials and methods. The first block of tissue culture wells of each cell type was treated with both agents, including increasing amounts of PS-341 (down arrow) plus increasing levels of TRAIL (right arrow). The second block of wells from each cell line was treated with these agents individually, increasing PS-341 concentrations (top row), TRAIL concentrations (middle row), or DMSO, the vehicle (bottom row)

are missing Bax, two colon and one prostate cancer cell line were examined. The parental colon cancer cell line HCT-116 expresses Bax protein and is highly sensitive to TRAIL-induced death, while the variant HC-4 cell line derived from HCT-116 contains a frame-shift mutation yielding cells that do not contain Bax protein and are resistant to TRAIL-induced cell death (Rampini *et al*., 1997). Using crystal violet staining as a means of detecting live cells, we incubated HC-4 and HCT-116 cells with these agents alone or in combination. The results demonstrated (Figure 2b) that the addition of either 0.1 or 1 μM PS-341 had no or minimal effect on these cells, whereas the addition of PS-341 enabled TRAIL to kill the HC-4 cells, and lowered the concentration of TRAIL necessary to kill HCT-116 cells. Like HC-4 cells, DU-145 human prostate cancer cells also contain a frame-shift mutation in Bax. These cells also demonstrated a combined response of TRAIL and PS-341 (data not shown).

To test whether PS-341 enhances TRAIL-mediated apoptosis by upregulating proteins in the TRAIL pathway, we examined the levels of specific proteins in the pathway after treatment with each agent alone or together. In LNCaP cells, PS-341 did not induce changes in the levels of FADD, Bak, or Bax (Figure 3a); or caspases 3, 8 or XIAP (data not shown). Although levels of caspase 8 and FADD were unchanged after PS-341 treatment, we found that overnight treatment of LNCaP cells with PS-341 and TRAIL resulted in increased cleavage of caspase 8, as evidenced by elevated levels of 41, 43, and 18 kDa cleavage products (Figure 3b). This increase in caspase 8 cleavage was associated with a decrease in levels of Bid (Figure 3b), a caspase 8 substrate, and activation of the effector caspases 3 and 7 (data not shown). Quantitation of these Western blots using scanning densitometry demonstrated a 50% increase in the levels of the 18 kD caspase 8 fragment with the combined treatment compared to treatment with TRAIL alone. Similar results were obtained with the JCA-1 human bladder cancer cell line (Figure 3c). The figure shows that in the presence of PS-341 the extent of caspase 8 and Bid cleavage is directly related to the dose of TRAIL applied.

Recent evidence suggests that c-FLIP plays an important role in TRAIL sensitivity, and that down-regulation of functional c-FLIP in response to doxorubicin treatment (Kelly *et al*., 2002), cell differentiation (Hietakangas *et al*., 2003), or phosphorylation (Higuchi *et al*., 2003) increases sensitivity to TRAIL-mediated apoptosis. In addition, FLIP is subject to ubiquitination and proteasomal degradation (Kim *et al*., 2002). To determine the role of FLIP in PS-341 action, we examined levels of c-FLIP-L and c-FLIP-S in HC-4 and LNCaP cells using an antibody that recognizes both forms of c-FLIP (Scaffidi *et al*., 1999). The results are

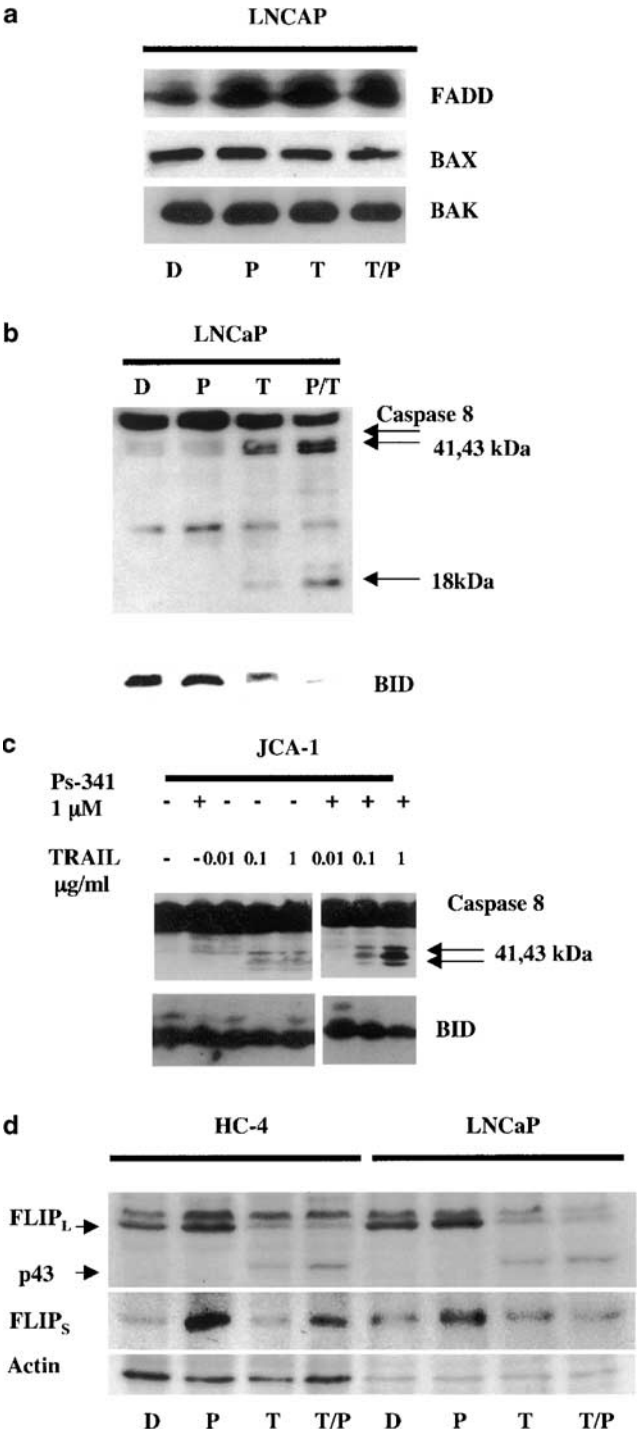

Figure 3 TRAIL and PS-341 treatment increase the levels of activated caspase 8 and cleaved Bid. (**a**) Levels of proapoptotic proteins in LNCaP cells. LNCaP cells were treated with vehicle (DMSO), PS-341 (1 μM), TRAIL (100 ng/ml), or both agents for 18 h, and extracts of these cells Western blotted with antibodies to BAX, BAK, and FADD. (**b**) Activation of caspase 8 by TRAIL and PS-341. LNCaP human prostate cancer cells were treated overnight with DMSO (D), PS-341 (1 μM) (P), TRAIL (100 ng/ml) (T), or a combination of both agents (T/P). (**c**) JCA-1 human bladder cancer cells were treated with varying concentrations of TRAIL and/or PS-341 (1 μM). Extracts were Western blotted with antibodies to either Bid or caspase 8. The arrows identify known caspase 8 cleavage products. (**d**) Response of cFLIP to PS-341 and TRAIL. HC-4 human colon cancer cells and LNCaP human prostate cancer cells were treated for 4 h (HC-4) or 6 h (LNCaP) with DMSO (D), PS-341(1 μM) (P), TRAIL(100 ng/ml), (T), or the combination (T/P). Cell extracts were Western blotted with NF6 antibody to cFLIP

shown in Figure 3d. In both cell lines, c-FLIP$_L$, and c-FLIP$_S$ appear upregulated in response to PS-341. In addition, the levels of the p43 cleavage product appear equivalent in samples treated with TRAIL or the combination of PS-341 and TRAIL. These data do not suggest a role for c-FLIP in PS-341-mediated sensitization to TRAIL.

It was shown that after treatment with TRAIL, HCT-116, but not HC-4 (Bax negative), cells responded with mitochondrial release of SMAC protein (Deng *et al*., 2002). However, HC-4 cells transfected with SMAC were able to undergo TRAIL-induced cell death, demonstrating that SMAC is sufficient for TRAIL-induced death in these cells (Deng *et al*., 2002). The addition of TRAIL alone to HC-4 and HCT-116 causes similar levels of caspase 8 and Bid cleavage (Deng *et al*., 2002, Figure 4a), suggesting that the difference in the ability of these cells to die may be regulated by levels of Bax. In contrast, others have shown a difference in the extent of cleavage of caspase 8 by TRAIL in these two cell lines (LeBlanc *et al*., 2002). As in LNCaP and JCA-1 cells, the combination of PS-341 and TRAIL induced increased cleavage of caspase 8 and Bid (Figure 4a). As expected, neither TRAIL nor PS-341 addition in isolation was sufficient to cause release of SMAC (Figure 4a, 16) or cytochrome *c* (data not shown; Deng *et al*., 2002) from HC-4 cells. However, the combination caused rapid release of SMAC, detectable in 1 h and with continued release up to 4 h (Figure 4b) after treatment.

The requirement for SMAC protein in TRAIL-induced death of HC-4 cells prompted us to ask whether PS-341 required cytochrome *c*-activated caspase 9 to potentiate TRAIL-induced death. For this purpose, we used caspase 9-deficient MEF (Hakem *et al*., 1998). Wild-type MEF release cytochrome *c* and SMAC in response to combination treatment with PS-341 and TRAIL (data not shown). To test whether the absence of caspase 9 would block the effect of combined agents, wild-type and caspase 9-deficient MEF were exposed for 18 h to TRAIL, PS-341, or the combination, and analysed for cell death by DAPI uptake. As shown in Figure 5, both wild-type and caspase 9-deficient MEF underwent apoptosis to a similar degree in response to combination treatment of PS-341 and TRAIL, indicating that caspase 9 is dispensable for the potentiating effect.

To evaluate whether increased activation of caspase 8 by TRAIL and PS-341 was sufficient to induce cell death, we transfected HC-4 and HCT-116 cells with a vector that expressed the catalytic portion of the caspase 8 molecule cloned in frame with three repeats of the FK506 binding protein (FKBP12) (Fkp-caspase 8) (Yang *et al*., 1998). Overexpression of this protein in the absence of crosslinking was sufficient to induce apoptotic cell death in 293 cells (data not shown), although crosslinking of the FK506 sequences enhances the ability of this protein to induce cell death (Yang *et al*., 1998). We used X-Gal staining of LacZ-transfected cells as a marker of those cells expressing

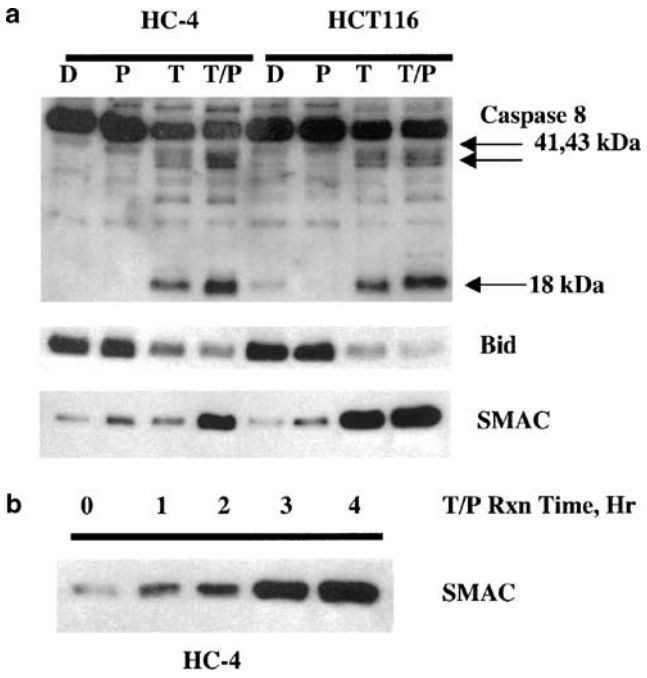

Figure 4 PS-341 enhances the ability of TRAIL to release SMAC from Bax-deficient HC-4 cells. (**a**) SMAC is released from HC-4 cells by the combination of TRAIL and PS-341. HC-4 and HCT-116 were treated for 3 h with DMSO (D), TRAIL (T) (100 ng/ml), PS-341 (P) (1 μM) or both (T/P) agents. Extracts of these cells were run on 12% SDS : PAGE gels and Western blotted with antibodies to the indicated proteins. (**b**) HC-4 cells rapidly release SMAC after the addition of TRAIL (100 ng/ml and PS-341 (1 μM) (T/P). HC-4 cells were treated with both compounds for varying periods of time as shown. Extracts of these cells were treated as in (**a**)

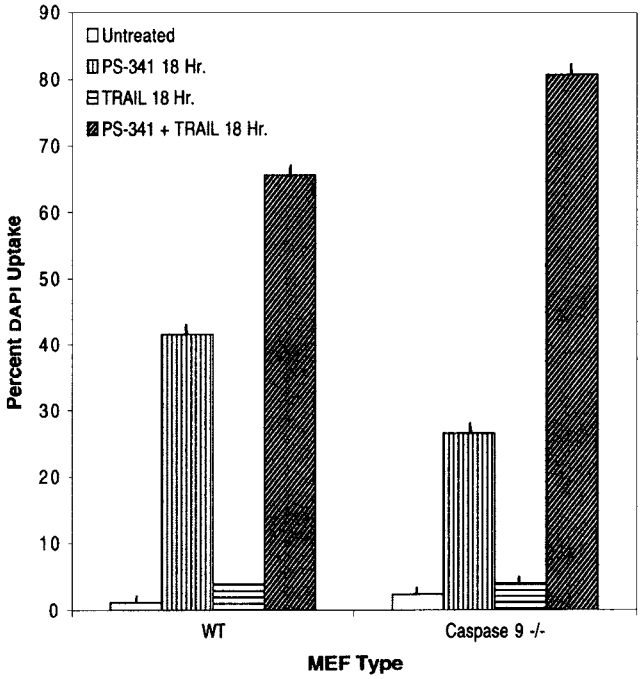

Figure 5 Response of caspase 9$^{-/-}$ MEF to PS-341 and TRAIL. Caspase 9$^{-/-}$ MEF were exposed to vehicle, 0.1 μM PS-341, 1 μg/ml TRAIL, or the combination for 18 h. Cells were processed for DAPI uptake as described in Materials and methods. Data are expressed as mean and range of two independent experiments

transfected Fkp-caspase 8. We found that the overexpression of activated caspase 8 in HC-4 cells was able to induce apoptosis and that the level of apoptosis, although slightly less than in HCT-116, was not significantly different than in this parental cell line (Figure 6). Thus, in the absence of Bax, increased levels of activated caspase 8 are sufficient to induce apoptosis of these colon cancer cells.

Since the combination of PS-341 and TRAIL increased caspase 8 activation, we evaluated the ability of PS-341 to affect the levels of TRAIL receptors DR5 and DR4. HC-4 cells were incubated with PS-341 alone for varying periods of time. The cells were then either incubated with histidine-tagged TRAIL and lysed to measure cell surface receptors, or lysed followed by addition of histidine-tagged TRAIL to measure whole-cell receptor levels. Both DR5 and DR4 receptors were then immunoprecipitated with antihistidine antibody. Controls were not treated with histidine-tagged TRAIL, although the rest of the procedure was followed. In contrast to PS-341's lack of effect on levels of TRAIL pathway proteins downstream from TRAIL receptors, incubation of HC-4 cells with PS-341 alone resulted in marked accumulation of TRAIL receptor DR5 and to a lesser extent DR4 both in whole-cell lysates and (as shown, Figure 7a) on the cell surface protein. After 3 h treatment with PS-341, approximately the time HC-4 cells with apoptotic morphology begin to appear in cultures treated with the combination of PS-341 and TRAIL, DR5 receptors are reproducibly elevated ($51 \pm 13\%$, $n = 4$), while 18 h treatment with PS-341 raised DR5 levels 4–8-fold. In comparison, DR4 levels did not increase appreciably by 3 h although an approximate doubling was observed after 18 h. Similar changes in DR5 and DR4 were obtained with treatment of PC-3 human prostate cancer cells with PS-341 (data not shown).

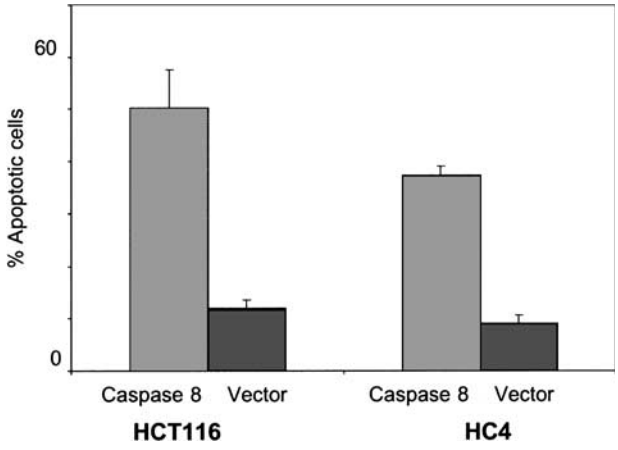

Figure 6 Overexpression of caspase 8 induces apoptosis in HC-4 and HCT-116 cells. HC-4 and HCT-116 were transfected with a plasmid containing encoding the LacZ cDNA with or without equivalent amounts of Fkp3-caspase 8 (180) or carrier DNA. After 24 h, the plates were stained with X-Gal. In all, 10 fields in each well were scored for total blue and apoptotic blue cells (approx. 200 total blue cells/well). The s.d. of the mean of two transfection experiments performed in triplicate is shown

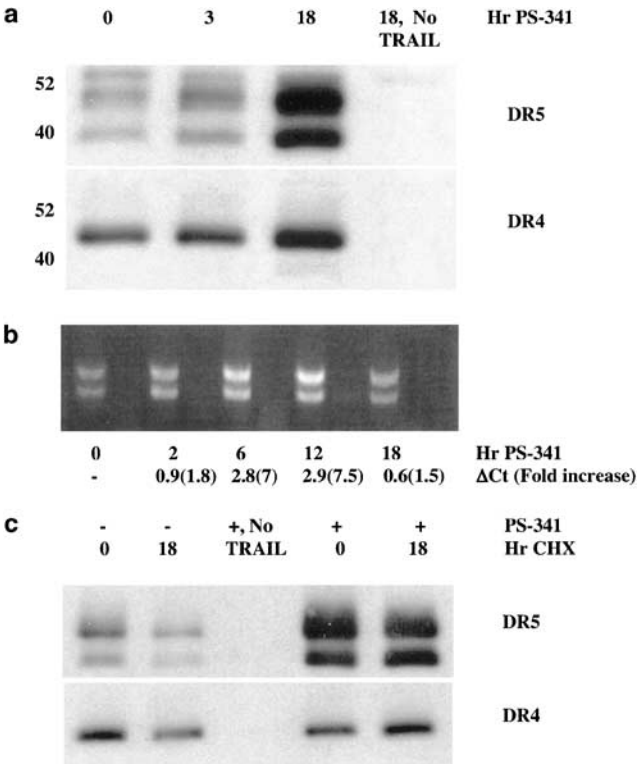

Figure 7 Response of TRAIL receptor protein and mRNA expression to PS-341 treatment. (**a**) PS-341 treatment increases cell surface DR5 and DR4 receptors. HC-4 cells were exposed to 1 μM PS-341 for 0, 3, and 18 h. Cell surface TRAIL receptors were prepared as described in Materials and methods and analysed by Western blotting for DR4 and DR5 protein. The lane labeled 'No TRAIL' represents cells to which histidine-tagged TRAIL was not added prior to cell lysis and incubation with antihistidine agarose (cf. Materials and methods). (**b**) DR5 transcript levels increase in response to PS-341 treatment. Semiquantitative PCR (gel) and quantitative real-time PCR (Δ Ct) were performed on cDNA prepared from RNA of HC-4 colon cancer cells exposed to PS-341 (1 μM) for various times. ΔCt values (the difference in cycle number at a given threshold during the linear phase of amplification) are relative to control cells; fold increases ($2^{\Delta Ct}$) are in parentheses. (**c**) PS-341 treatment prevents degradation of DR4 and DR5 protein. HC-4 cells were exposed to vehicle or PS-341 for 6 h. At this point, cycloheximide (50 μM) was added and incubation was continued for an additional 18 h. Total cellular TRAIL receptors were isolated as described in Materials and methods and subjected to Western analysis for DR4 and DR5. The lane labeled 'No TRAIL' is as in (**a**)

To determine whether the observed increases in these proteins were due to transcriptional activation, semi-quantitative and quantitative real-time PCR was performed as described in Materials and methods. This technique was chosen over Northern analysis to avoid complications arising from the high degree of sequence similarity between the receptors (MacFarlane *et al*., 1997). Figure 7b shows semiquantitative RT–PCR of cDNA from HC-4 cells exposed to PS-341 for varying amounts of time, using primers specific for DR5. The same cDNAs were subjected to quantitative real-time PCR and the ΔCt values (relative to cDNA from control cells) are indicated beneath the corresponding lanes. The results indicate an early increase in DR5 mRNA

transcripts of about twofold, consistent with the observed rise in DR5 protein, with maximal increases of 6–8-fold at 6 and 12 h followed by a return to baseline.

To address the possibility that PS-341 increased protein stability and thus contributed to accumulation of DR4 and DR5, receptor levels were determined following exposure of HC-4 cells to cycloheximide. An example of such an experiment is shown in Figure 7c. In the presence of PS-341, DR5 levels were unchanged after 18 h of cycloheximide treatment; in the absence of PS-341, levels decreased by 2.5-fold, suggesting that PS-341 treatment prevented the degradation of this protein. Attempts to determine the half-life of DR4 and DR5 by pulse-chase experiments utilizing ^{35}S-methionine proved difficult to interpret due to nonspecific binding of labeled proteins to the antihistidine antibody in the critical molecular weight range. Thus, our data suggest transcriptional activation at early time points, and protein accumulation secondary to decreased degradation.

The increased stability of DR5 in the presence of PS-341 prompted us to examine whether this receptor might be ubiquitinated. To investigate this possibility, a FLAG-tagged DR5 construct containing an inactivating mutation in the death domain to prevent the induction of apoptosis was cotransfected into 293 T cells with a cDNA expressing HA-tagged ubiquitin. Following treatments, DR5 was recovered by immunoprecipitation with anti-FLAG antibody coupled to agarose beads and analysed by Western blotting. The results are shown in Figure 8. High molecular weight forms of DR5 were detected with antibodies against HA, and were greatly increased by treatment with PS-341, thus demonstrating the enhanced ubiquitination of this protein.

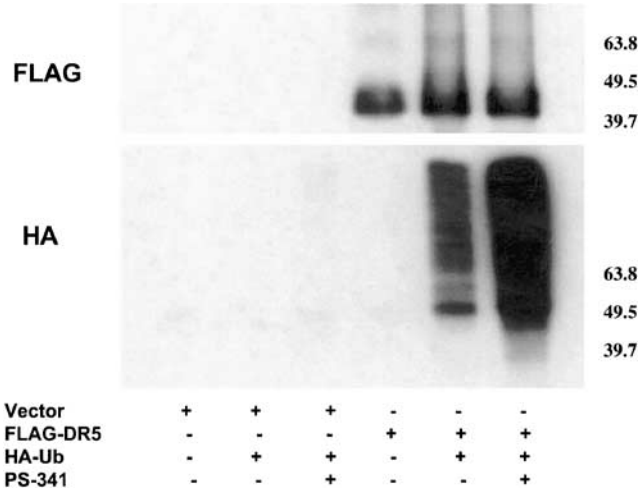

Figure 8 Ubiquitination of DR5. Expression vectors containing FLAG-tagged DR5 (L334N) (FLAG-DR5), HA-tagged ubiquitin (HA-Ub), or empty vector were transfected into 293 T cells in the combinations indicated. After 24 h, PS-341 (1 μM) or vehicle was added and the cultures were incubated overnight. Cell lysates were incubated overnight with anti-FLAG antibody covalently coupled to agarose beads at 4°. Bound material was eluted and prepared for Western blotting as described for DR5 immunoprecipitation. Blots were stained for DR5 (FLAG) or ubiquitin (HA) as indicated

Since HC-4 cells are transformed human colon cancer cells and might contain additional mutations in the apoptotic pathway in addition to the lack of Bax, we wanted to assess the sensitivity of normal cells, also devoid of Bax, to this combination of agents. In addition, it was reported that either Bax or Bak can mediate sensitivity to tBid-induced apoptosis (Wei *et al*., 2001), suggesting that cells devoid of Bak should have a similar phenotype to those that were Bax-negative. We

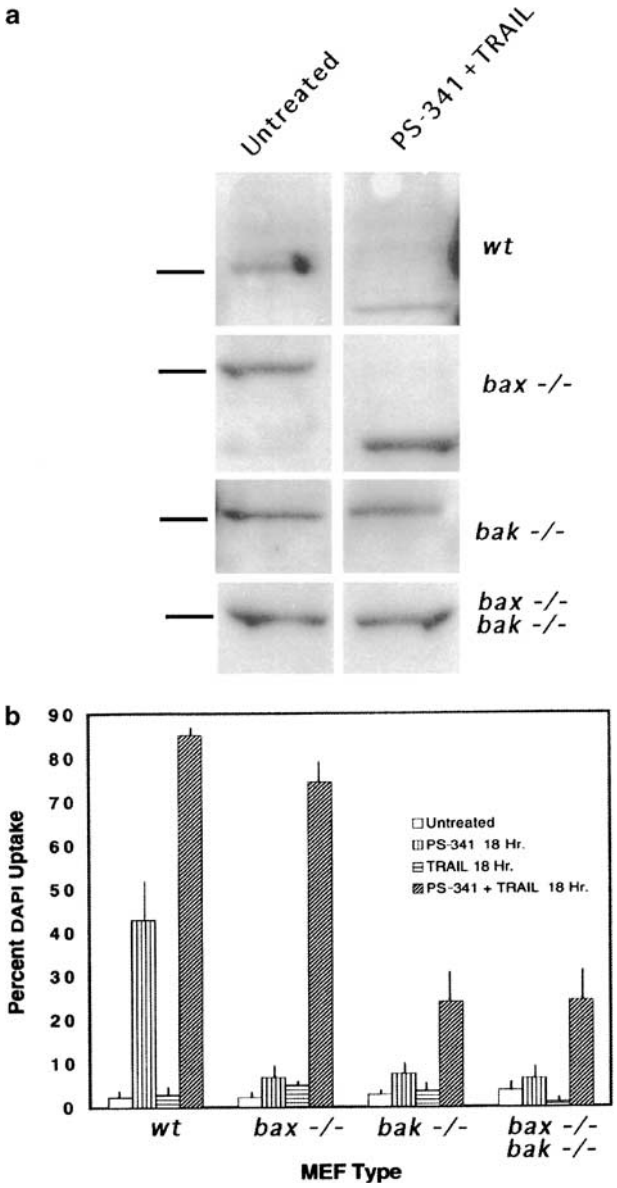

Figure 9 MEF response to PS-341 and TRAIL. (**a**) Combined treatment causes PARP cleavage in *wt* and *bax*$^{-/-}$ but not *bak*$^{-/-}$ MEF. MEF of the indicated genotype were exposed to vehicle, or the combination treatment of 0.1 μM PS-341, 1 μg/ml TRAIL for 6 h. Cells were lysed and extracts analysed for PARP expression and cleavage by Western blotting. The dark line at left indicates the position of uncleaved PARP (116 kd). (**b**) *Bak*$^{-/-}$ cells are less sensitive to combined treatment with PS-341 and TRAIL. MEF of the indicated genotype were exposed to vehicle, 0.1 μM PS-341, TRAIL, or the combination for 18 h. Cells were processed for DAPI uptake as described in Materials and methods. Data are expressed as mean and range of two independent experiments

evaluated the response of MEF deficient in one or both of these proteins to PS-341, TRAIL, or the combination. Induction of apoptosis was evaluated by estimating the amount of PARP cleavage after 6 h treatment (Figure 9a) or by uptake of the fluorescent dye DAPI after 18 h treatment (Figure 9b). A total of 6 h was chosen to evaluate PARP cleavage, because by 18 h a large number of cells were apoptotic. After 6 h, PARP cleavage is essentially complete in wild-type (*wt*) and *bax*$^{-/-}$ MEF treated with the combination of PS-341 and TRAIL. At this time, less than 5% of *wt* and *bax*$^{-/-}$ cells took up DAPI irrespective of treatment. After 18 h treatment with the combination of PS-341 and TRAIL, 75% or more *wt* and *bax*$^{-/-}$ cells took up DAPI and demonstrated an apoptotic nuclear morphology. In contrast to the data obtained with *bax*$^{-/-}$ fibroblasts, at 6 h in the bak$^{-/-}$ or *bax*$^{-/-}$ *bak*$^{-/-}$ cells, there was no evidence of PARP cleavage in cells treated with the combination of PS-341 and TRAIL; and only 25% of *bak*$^{-/-}$ or *bax*$^{-/-}$ *bak*$^{-/-}$ cells underwent apoptosis after 18 h as evidenced by DAPI uptake. Thus, cells lacking Bak appear to be significantly more resistant to combination treatment with PS-341 and TRAIL.

Discussion

The ability of PS-341 to enhance TRAIL-induced cell death is not tumor-type specific and was seen by us in LNCaP cells; two additional prostate cancer cell lines, DU-145 and PC-3; in the bladder cancer cell lines JCA-1 and TSU-PR (Van Bokhoven *et al*., 2001); in the colon cancer cell line HC-4; and in MEF. The ability of proteasome inhibitors, including PS-341, to stimulate TRAIL-induced apoptosis has also been reported in other cell lines (Mitsiades *et al*., 2001; Franco *et al*., 2001).

The effects of TRAIL on induction of apoptosis in human prostate cancer cells are known to be blocked by overexpression of Bcl-2 or Bcl-xL (Munshi *et al*., 2001; Roklin *et al*., 2001). However, we found that the combination of PS-341 and TRAIL overcame the block to mitochondrial participation in the apoptotic process mediated by overexpression of Bcl-xL. We obtained similar results in cells deficient in Bax or caspase 9. The mechanism allowing this effect appears to originate in part with the ability of PS-341 to increase the levels of both DR5 and DR4 receptors, leading to enhanced cleavage of caspase 8 and Bid, and increased release of SMAC and cytochrome *c*. Since this combination of agents is active in caspase 9-deficient MEF, it is likely that the apoptosome including cytochrome *c*, APAF-1, and caspase 9 is not needed for this effect and activated caspase 8 cleaves caspases 3 and 7 directly leading to the induction of apoptosis. Other agents, including sulindac (He *et al*., 2002), and chemotherapeutic agents etoposide and camptothecin (Gibson *et al*., 2000; Nagane *et al*., 2000; Lacour *et al*., 2001; LeBlanc *et al*., 2002), have been shown to enhance the ability of TRAIL to kill Bax-negative HC-4 cells (LeBlanc *et al*., 2002) presumably by inducing increases in DR5 but not DR4 TRAIL receptors. Quantitative PCR indicates that PS-341 induces a twofold increase in mRNA for DR5 at 2 h after treatment of HC-4 cells, increasing to eightfold by 12 h, suggesting that PS-341 controls the levels of DR5 in part through transcriptional mechanisms. Array experiments carried out by others after chemotherapy treatment of HC-4 cells have shown a similar level of increase in DR5 (LeBlanc *et al*., 2002), but not DR4, while PS-341 treatment of myeloma cells caused no change in mRNA levels at 2 and 4 h but did induce an increase at 8 h (Mitsiades *et al*., 2002).

The ubiquitin–proteasome pathway acts as a regulator of endocytosis of selected membrane receptors, for example, the growth hormone and epidermal growth factor receptors, and controls lysosomal degradation (Govers *et al*., 1999; Longva *et al*., 2002) of these receptors. It was shown that degradation of many ubiquitinated membrane proteins was slowed in the presence of proteasome inhibitors (van Kerkhov and Strous, 2001) and that mutational inactivation of the proteasome pathway prevented endocytosis of the growth hormone receptor and increased its abundance on the cell surface (van Kerkhov *et al*., 2002). DR5 is known to be internalized into endosomes following exposure of cells to TRAIL (Zhang *et al*., 2000). Our findings that DR5 is ubiquitinated and that PS-341 increased its stability suggest that similar mechanisms may be involved for these death receptors. Thus, PS-341 may enhance the number of TRAIL receptors both through protein stabilization and transcriptional mechanisms.

The effect of PS-341 on c-FLIP is of interest due to accumulating evidence of the importance of c-FLIP in receptor-mediated apoptosis. Both c-FLIP$_L$ and c-FLIP$_S$ have been shown to inhibit receptor-mediated apoptosis, apparently by different mechanisms (reviewed in Krueger *et al*., 2001). Our data indicate that PS-341 does not downregulate levels of either form of FLIP, and that the combination of PS-341 and TRAIL does not alter the degree of c-FLIP$_L$ cleavage over that induced by TRAIL alone, even though caspase 8 cleavage is increased (Figures 3b,c and 4a), It could be that FLIP is limiting while caspase 8 is not, thus allowing for greater caspase 8 activation in response to PS-341-induced upregulation of TRAIL receptors. Other mechanisms are possible, for example, c-FLIP phosphorylation (Higuchi *et al*., 2003).

Experiments examining the ability of PS-341, TRAIL, or the combination of these agents to induce apoptosis in MEF missing Bax, Bak, or both proteins suggests an important role for these proteins in regulating apoptosis induced by these agents. Interestingly, wild-type MEF underwent moderate levels of apoptosis when incubated with PS-341 alone, but MEF missing either Bax or Bak were resistant. The mechanism by which PS-341 induces apoptosis is unknown. Suggestions have been made that modulation of NF-κB plays a role in its action (Wang *et al*., 1998). Recent array data (Mitsiades *et al*., 2002) suggest that PS-341 upregulates the levels of a number of caspases and decreases the levels of Bcl-2. Clearly, from these data the mitochondrial death pathway is

necessary for PS-341-induced apoptosis. It is possible that PS-341 treatment activates a BH3 protein that binds to Bak or Bax to induce mitochondrial release of cytochrome *c* and other proapoptotic proteins.

We found that treatment with the combination of TRAIL and PS-341, as opposed to treatment with these compounds individually, triggered apoptosis much more effectively in MEF containing Bak. The combined treatment was significantly less effective in MEF lacking Bak, or both Bak and Bax. One explanation for our results is that a PS-341-induced increase in Bak protein is needed to enhance TRAIL-mediated apoptosis. The addition of etoposide to HC-4 cells was shown to stimulate a marked increase in Bak mRNA, although the actual protein levels were not measured (LeBlanc *et al*., 2002). However, we observed no increase in Bak protein with PS-341 treatment in multiple cell types including LNCaP, HC-4, and MEF (data not shown). Whether the functions of Bak and Bax are completely interchangeable is not known. Bak-deficient T cells were resistant to apoptosis induced by staurosporine, etoposide, and bleomycin (Wang *et al*., 2001) and failed to release cytochrome *c* when transfected with Bid. Mandic *et al*. (2001) found that cisplatin induced the proapoptotic conformation of Bak, but not of Bax, in three out of four melanoma cell lines, suggesting some specificity in signaling to Bak. However, in baby mouse kidney cells, the presence of either Bak or Bax was sufficient to allow for TNF α-induced apoptosis (Degenhardt *et al*., 2002). Both Bax or Bak-deficient MEF were sensitive to the killing effects of tBid (Wei *et al*., 2001), the activated form of Bid, and BimS (Zong *et al*., 2001), suggesting that these proteins were interchangeable in inducing the death pathway. It is possible that PS-341 regulates a BH3 domain protein which preferentially binds to Bak to induce apoptosis. Further experiments are needed to clarify this hypothesis.

Our data suggest a model whereby PS-341 enhances TRAIL killing by increasing the level of DR5 and DR4 receptors, thus increasing caspase 8 activation. As suggested by the induction of apoptosis in *bax*$^{-/-}$ *bak*$^{-/-}$ and caspase 9-deficient MEF, mitochondria may not be necessary for apoptosis induced by PS-341 and TRAIL; caspase 8 may directly cleave caspases 3 and 7 causing cell death. In the presence of Bak, however, the mitochondria are activated to release cytochrome *c*, SMAC, and other proteins that serve to amplify the signal from TRAIL receptors. The observation that caspase 9-deficient MEF are still responsive to this combination is consistent with previous experiments (Deng *et al*., 2002), suggesting that SMAC upregulation alone is sufficient for increased sensitivity to TRAIL. SMAC would function to further increase the activation of caspases 3 and 7.

Our data demonstrate that the combination of PS-341 and TRAIL should be active in treating human tumors with multiple abnormalities in the apoptotic cascade.

Abbreviations

TRAIL, TNF-like apoptosis-inducing ligand; DISC, death-inducing signaling complex; PARP, polyADP ribose polymerase; MEF, murine embryonic fibroblasts.

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Elevated Akt Activity Protects the Prostate Cancer Cell Line LNCaP from TRAIL-induced Apoptosis*

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We find that the prostate cancer cell lines ALVA-31, PC-3, and DU 145 are highly sensitive to apoptosis induced by TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand), while the cell lines TSU-Pr1 and JCA-1 are moderately sensitive, and the LNCaP cell line is resistant. LNCaP cells lack active lipid phosphatase PTEN, a negative regulator of the phosphatidylinositol (PI) 3-kinase/Akt pathway, and demonstrate a high constitutive Akt activity. Inhibition of PI 3-kinase using wortmannin and LY-294002 suppressed constitutive Akt activity and sensitized LNCaP cells to TRAIL. Treatment of LNCaP cells with TRAIL alone induced cleavage of the caspase 8 and XIAP proteins. However, processing of BID, mitochondrial release of cytochrome *c*, activation of caspases 7 and 9, and apoptosis did not occur unless TRAIL was combined with either wortmannin, LY-294002, or cycloheximide. Blocking cytochrome *c* release by Bcl-2 overexpression rendered LNCaP cells resistant to TRAIL plus wortmannin treatment but did not affect caspase 8 or BID processing. This indicates that in these cells mitochondria are required for the propagation rather than the initiation of the apoptotic cascade. Infection of LNCaP cells with an adenovirus expressing a constitutively active Akt reversed the ability of wortmannin to potentiate TRAIL-induced BID cleavage. Thus, the PI 3-kinase-dependent blockage of TRAIL-induced apoptosis in LNCaP cells appears to be mediated by Akt through the inhibition of BID cleavage.

TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) (1) also known as Apo-2 ligand (2) is a proapoptotic cytokine that together with three related proteins (tumor necrosis factor-α, CD95L/FasL, and TWEAK/Apo3L) constitutes a family of ligands that transduce death signals through death domain-containing receptors (3–5). TRAIL is a type II transmembrane protein that functions by binding to two closely related receptors, DR4 and DR5 (6). Both TRAIL and its receptors are ubiquitously expressed (7), suggesting the existence of mechanisms that protect normal tissues from TRAIL-induced apoptosis.

TRAIL is capable of inducing apoptosis in a wide variety of cancer cells in culture and in tumor implants in mice, including cancers of the colon, breast, lung, kidney, central nervous system, blood, and skin (1, 6, 8–11). At the same time, unlike tumor necrosis factor-α and Fas ligand, whose use for cancer therapy has been hampered by their severe toxicity (12, 13), TRAIL has no toxic effects when systemically administered in rodents (10) and nonhuman primates (9). Although the majority of normal human cells tested so far appear to be TRAIL-resistant, recent experiments have demonstrated that cultured human liver cells may be sensitive to TRAIL (14), suggesting that additional studies are required to investigate what determines resistance or sensitivity to this agent.

Despite the ubiquitous expression of TRAIL receptors, a significant proportion of cell lines originating from various cancer types demonstrate either partial or complete resistance to the proapoptotic effects of TRAIL. These findings suggest either defects in apoptotic pathways or the presence of inhibitors of TRAIL-induced apoptosis. The latter possibility appears to be more likely, since the resistance of many types of cancer cells to TRAIL can be reversed by treatment with protein synthesis inhibitors (15–19) or chemotherapeutic agents (9, 11). Some normal human cells can also be sensitized to TRAIL by the inhibition of protein synthesis (20). The elucidation of mechanisms that control sensitivity to TRAIL may lead to better understanding of death receptor-mediated signaling and help to develop TRAIL-based approaches to cancer treatment.

Activation of death receptors leads to the formation of the death-inducing signaling complex (DISC)[1] (21), which includes the receptor itself, and caspase 8 (22). The recruitment of caspase 8 to TRAIL receptors DR4 and DR5 is thought to be mediated by the adaptor protein FADD (23–25). The formation of the DISC triggers autoprocessing and activation of caspase 8 (22) that in turn results in the cleavage and activation of the effector caspase 3 or 7 (26, 27), leading to apoptosis. Activated caspase 8 may also cleave a proapoptotic protein BID, whose cleavage product triggers cytochrome *c* release from mitochondria (28, 29). In some but not all cell types, the mitochondrial step may be required to amplify the apoptotic signal and fully activate caspase 8 (30). Since the TRAIL-induced apoptotic signal is a multistep process, inhibition of this cascade may occur at several stages. For example, at the ligand-receptor level, TRAIL signaling could be inhibited by the overexpression of nonfunctional TRAIL receptors DcR1 or DcR2 (31) or by proteins that induce rapid internalization of TRAIL receptors

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[1] The abbreviations used are: DISC, death-inducing signaling complex; PI, phosphatidylinositol; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

(similar to Fas inhibition the adenoviral protein E3) (32). At the DISC, the apoptotic pathway may be inhibited by cFLIP protein that is capable of blocking processing and activation of caspase 8 (33, 34). Downstream of DISC, IAP proteins may specifically inhibit the executor caspases 3 and 7 (35). In those cells that require mitochondria to stimulate apoptosis, the signal may be inhibited by Bcl-2/Bcl-X_L types of proteins that prevent the release of proapoptotic factors from the mitochondria (30).

In the present study, we tested the cytotoxic effects of TRAIL on six human prostate cancer cell lines, demonstrating variable responses, with some cell lines being extremely sensitive and others highly resistant. The highly resistant cell line LNCaP was further investigated to examine mechanisms that protect it from TRAIL-mediated apoptosis. We find that the TRAIL-induced death signal in LNCaP cells is negatively regulated by a high constitutive activity of protein kinase Akt. Furthermore, the antiapoptotic block occurs downstream of caspase 8 activation at the level of BID protein cleavage. This study is the first demonstration that the PI 3-kinase/Akt pathway may interfere with an apoptotic signal by inhibiting processing of BID.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies were obtained from the following sources: anti-phospho-Akt (New England Biolabs, Beverly, MA); anti-cytochrome *c* and anti-BID (Zymed Laboratories Inc.); anti-Akt and anti-XIAP (Transduction Laboratories, Lexington, KY); anti-HA1 tag (Babco, Richmond, CA); anti-caspase 8 (Upstate Biotechnology, Inc., Lake Placid, NY); anti-caspase 7 (PharMingen, San Diego, CA); anti-caspase 9 (Oncogene Research Products, Boston, MA); anti-$FLIP_L$ (Affinity BioReagents, Golden, CO); anti-FLIPγ/δ (Calbiochem).

Cell Culture—Prostate cancer cell lines LNCaP, PC-3, DU 145, TSU-Pr1, JCA-1, and ALVA-31 were passaged in RPMI 1640 with 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin. The sources for these cell lines, their characterization, and use in our laboratories have been described previously (36). LNCaP cells overexpressing Bcl-2 (37) were kindly provided by Dr. R. Buttyan (Columbia Presbyterian Medical Center, New York, NY) and grown in medium supplemented with 400 μg/ml of G418.

Expression of Recombinant TRAIL in Yeast Pichia pastoris—A cDNA encoding for soluble human TRAIL (residues 114–281) was amplified by polymerase chain reaction from the expressed sequence tag clone 117926 (GenBank™ accession number T90422) in frame with the N-terminal hexahistidine tag using oligonucleotides 5′-AGTCATGAATTCCATCACCATCACCATCACGTGAGAGAAAGAGGTCCTCAGAGAGTAG-3′ and 5′-AGTCATGGTACCTTAGCCAACTAAAAAGGCCCCGAAAAA-3′. This cDNA was then cloned into the *Eco*RI/*Kpn*I sites of pPICZαA vector (Invitrogen, Carlsbad, CA) in frame with the cleavable secretion signal from yeast α factor. All manipulations of yeast were performed in general as outlined in the Invitrogen manual. Briefly, the expression vector was linearized and transformed by electroporation into *P. pastoris* strain SMD1168 (38). Transformants were selected on 500 μg/ml of Zeocin, and secretion of TRAIL was tested by Western blotting. For large scale production, yeast were grown for 24 h in 10 liters of complex medium containing glycerol and antifoam 289 (Sigma, St. Louis, MO) and buffered with 100 mM potassium phosphate buffer, pH 6.0, at constant aeration and mixing to A_{600} of 15. To induce TRAIL production, cells were pelleted by centrifugation, resuspended in complex medium containing 0.5% methanol, and grown for 24 h. The supernatant was concentrated using tangential flow Prep/Scale-TFF cartridge (Millipore Corp., Bedford, MA) and recombinant TRAIL purified by nickel-chelate chromatography on a Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen, Valencia, CA). This procedure yielded about 2 mg of pure protein from 1 liter of yeast supernatant.

Cytotoxicity Assays—Cell viability was determined spectrophotometrically using an Aqueous One tetrazolium-based assay (Promega, Madison, WI). Absorbance was measured at 490 nm, and data from duplicate determinations were plotted as percentage of untreated control cells. Quantitative analysis of DNA fragmentation was done using a Cell Death Detection ELISA^{plus} kit (Roche Diagnostics Corp., Indianapolis, IN) by measuring relative amounts of DNA-histone complexes released into the cytoplasm. Data from triplicate determinations were plotted as percentage of control of untreated cells. A TUNEL assay was performed using the FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA).

Measurement of Cytochrome c Release from Mitochondria—Cytosolic extracts from LNCaP cells were prepared by the hypotonic lysis procedure originally described by Bossy-Wetzel *et al.* (39) and modified by Carson *et al.* (40). LNCaP cells grown on 15-cm plates to 50% confluence were placed on ice and then scraped directly into growth medium and centrifuged for 2 min at 200 × *g*. Cell pellets were then washed once with ice-cold phosphate-buffered saline and resuspended in 300 μl of hypotonic lysis buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol) containing protease inhibitors, including Complete Mixture (Roche Molecular Biochemicals, Germany), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. Cells were incubated on ice for 45 min and homogenized by pipetting (10 passes up and down). Supernatants were cleared by 10-min centrifugation at 1000 × *g*, followed by 30 min at 100,000 × *g* and analyzed by Western blotting with the anti-cytochrome *c* antibody.

Construction of Adenoviral Vectors Expressing myr-Akt—The full-length coding sequence of human Akt1 was fused in frame with the myristoylation signal from the human Src protein in the N terminus and HA tag in the C terminus (myr-Akt). Kinase-dead construct was created by mutating lysine 179 for alanine, destroying in that way an ATP-binding site (myr-Akt(K−)). Recombinant adenoviruses were constructed by the method described by Crouzet *et al.* (41). Briefly, cDNAs of interest were subcloned into the expression cassette in plasmid vector pXL2996 under the control of the cytomegalovirus promoter. Each expression cassette was subcloned into the shuttle vector pXL3474. The resulting shuttle plasmids were introduced into *Escherichia coli* JM83 cells by electroporation. After double homologous recombinations, plasmid DNA for recombinant virus was purified by CsCl density gradient centrifugation. This DNA was linearized and transfected into 293 cells. 2–3 weeks after transfections, recombinant adenovirus was harvested from the conditioned medium and amplified in 293 cells.

RESULTS

Effect of Soluble TRAIL on Six Prostate Cancer Cell Lines—Recombinant human TRAIL (residues 114–281) was produced in methylotrophic yeast *P. pastoris* as a fusion protein containing an N-terminal hexahistidine tag and a cleavable secretion signal from yeast α factor. These features allowed quick one-step purification of secreted 20-kDa TRAIL by nickel-chelate chromatography from yeast supernatant yielding ~2 mg of pure protein from each liter of yeast culture medium (Fig. 1*A*). The cytotoxic effects of TRAIL were tested on a panel of six prostate cancer cell lines (Fig. 1*B*). Cell viability assays demonstrated that three of these cell lines, ALVA-31, DU 145, and PC-3 were very sensitive to TRAIL, JCA-1, and TSU-Pr1 revealed moderate sensitivity, whereas LNCaP cells were resistant to as high as 4 μg/ml of TRAIL. Internuclosomal fragmentation (DNA laddering) confirmed that cell death occurred by apoptosis (data not shown).

To investigate the mechanisms controlling the resistance of LNCaP cells to the cytotoxic effect of TRAIL, a series of Western and Northern blot experiments were done to compare the expression of various components of the TRAIL signaling pathway among the six prostate cancer cell lines. However, no correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4 and DR5, decoy receptors for TRAIL DcR1 and DcR2, initiator caspase 8, and apoptosis inhibitory protein cFLIP (data not shown). LNCaP cells contain a deactivating frameshift mutation in the gene encoding the tumor suppresser PTEN (42). This dual specificity phosphatase cleaves D3 phosphate of second messenger lipid phosphatidylinositol (PI) 3,4,5-trisphosphate (43). PI 3,4,5-trisphosphate produced by PI 3-kinase activates protein kinase Akt, and therefore, the lack of negative regulation by PTEN results in the constitutive activation of Akt in LNCaP cells (40). Immunoblot analysis with an antibody that specifically recognizes the phosphorylated/activated form of Akt (Ser^{473}) demonstrates that LNCaP cells possess the highest Akt activity among the six prostate cancer cell lines (Fig. 2*A*). Treating cells with the inhibitor of PI 3-kinase, wortmannin (200 nM), for 6 h

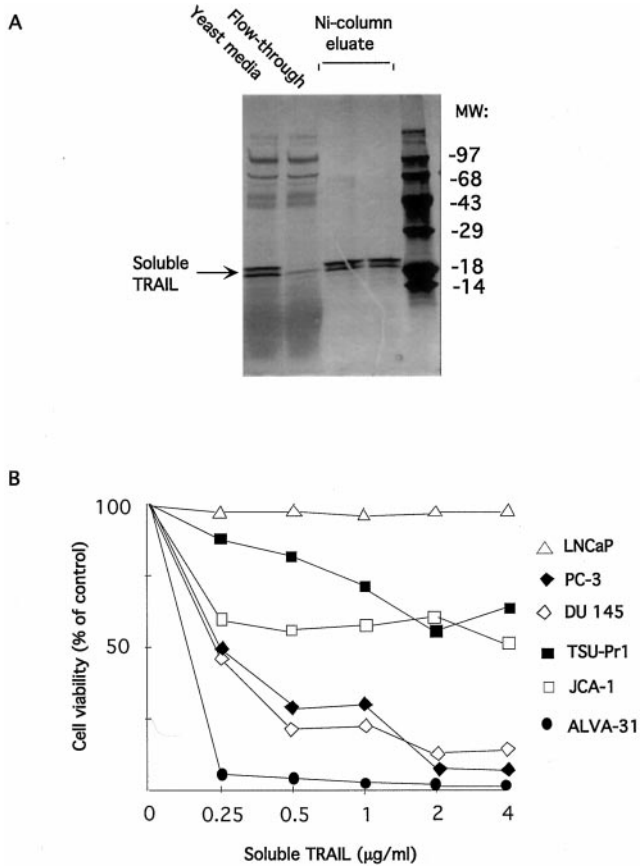

FIG. 1. **Sensitivity of human prostate cancer cell lines to soluble human TRAIL.** *A*, purification of recombinant TRAIL from *P. pastoris* supernatant by nickel-chelate chromatography. *B*, relative viability of six prostate cancer cell lines treated for 24 h with TRAIL, as measured by the tetrazolium conversion assay. Data are expressed as the means for duplicate determinations.

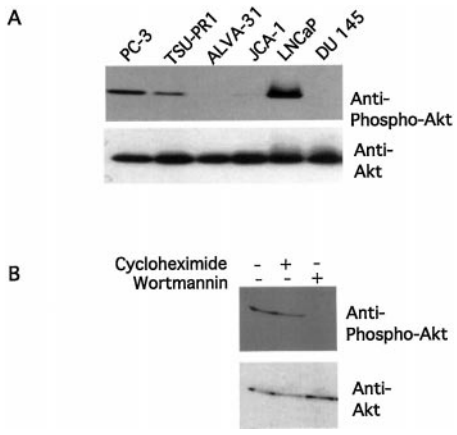

FIG. 2. **Constitutive activity of Akt in prostate cancer cells determined by immunoblot with anti-phospho-Akt antibody (Ser^{473}).** *A*, cell lysates prepared from six prostate cancer cell lines were probed by immunoblotting with anti-phospho-Akt antibody (*top panel*) or anti-Akt antibody (*bottom panel*). *B*, LNCaP cells were treated with wortmannin (200 nM) or cycloheximide (10 μM) for 6 h, and cell lysates were immunoblotted with anti-phospho-Akt antibody (*top panel*) or anti-Akt/PKBα antibody (*bottom panel*).

reverses the high constitutive activity of Akt (Fig. 2*B*).

Inhibition of PI 3-Kinase Activity or Protein Synthesis Renders LNCaP Cells Sensitive to TRAIL—To test whether the high constitutive activity of Akt in LNCaP cells results in their resistance to TRAIL, we first examined how PI 3-kinase inhibitors wortmannin (200 nM) and LY-294002 (20 μM) effect TRAIL cytotoxicity. Wortmannin acts at nanomolar concentrations by covalently modifying PI 3-kinase (44) but is unstable in aqueous solutions (45), making it possible that some PI 3-kinase activity can be restored by *de novo* synthesis in the course of the experiment. LY-294002 does not bind the enzyme covalently and has an IC_{50} value for PI 3-kinase about 500-fold higher than that of wortmannin (46) but is much more stable in culture medium. We have found that both substances significantly enhanced the proapoptotic activity of TRAIL in LNCaP cells as judged by apoptotic morphology (Fig. 3*A*) and DNA fragmentation (Fig. 3*B*), quantitated by measuring the relative amounts of DNA-histone complexes released into cytoplasm. Since wortmannin and LY-294002 inhibit PI 3-kinase by different mechanisms, this result confirms that sensitization of cells to TRAIL occurs through the inhibition of the PI 3-kinase pathway. Inhibition of protein synthesis with cycloheximide also sensitized LNCaP cells to TRAIL (Fig. 3, *A* and *B*). The DNA fragmentation induced by TRAIL in combination with wortmannin, LY-294002, or cycloheximide was greater than that triggered by the potassium ionophore valinomycin (Fig. 3*B*), a potent inducer of apoptosis (47). Thus, the resistance of LNCaP cells to TRAIL results from the blockage of the TRAIL-induced apoptotic signal transduction cascade rather than the defects in apoptotic machinery. These data demonstrate that the inhibition of TRAIL-mediated apoptosis in LNCaP cells requires PI 3-kinase activity and involves some short lived protein component(s).

TRAIL-mediated Cytochrome c Release Is Blocked in LNCaP Cells—Depending on the cell type, apoptotic signaling mediated by CD95/Fas may or may not require the release of proapoptotic factors (cytochrome *c* and apoptosis-inducing factor) from mitochondria. In type II, but not in type I cells, inhibition of mitochondrial apoptogenic activities by overexpression of Bcl-2 protein blocks Fas-mediated apoptosis (30). To examine whether the apoptogenic activity of mitochondria is required for the transduction of the TRAIL-induced death signal in LNCaP cells, the cytotoxic effects of TRAIL alone or in combination with wortmannin were studied in an LNCaP cell line overexpressing Bcl-2 (37). Quantitation of apoptotic nuclei by the TUNEL technique clearly demonstrates that Bcl-2 overexpression impairs the cytotoxic effect of TRAIL (Fig. 4*A*), indicating that mitochondria play an important role in TRAIL-induced apoptosis of LNCaP cells. If the resistance of LNCaP cells to TRAIL results from the high constitutive activity of Akt, this enzyme may block apoptosis either upstream (48, 49) or downstream (50) of mitochondrial cytochrome *c* release. To discriminate between these two possibilities, experiments were done to examine whether TRAIL-induced cytochrome *c* release is inhibited in LNCaP cells. LNCaP cells were incubated for 6 h with TRAIL alone or TRAIL in combination with cycloheximide or wortmannin. Cytosolic extracts were then prepared under conditions that keep mitochondria intact (39), and cytochrome *c* released to the cytosolic fraction was then detected by immunoblotting (Fig. 4*B*). This experiment demonstrated that in LNCaP cells TRAIL alone does not trigger the release of cytochrome *c* from the mitochondria, but it does so in combination with wortmannin and, to a lesser extent, cycloheximide. Thus, TRAIL-induced apoptotic signaling in LNCaP cells is blocked upstream of the mitochondria.

TRAIL-induced Apoptotic Signaling in LNCaP Cells Is Blocked at the Level of BID Cleavage—To understand at what biochemical step the TRAIL-mediated apoptotic cascade is blocked in LNCaP cells, a series of immunoblotting experiments were carried out using antibodies to proteins involved in this cascade. Our results demonstrate that processing of initiator caspase 8 is induced by TRAIL alone as efficiently as when

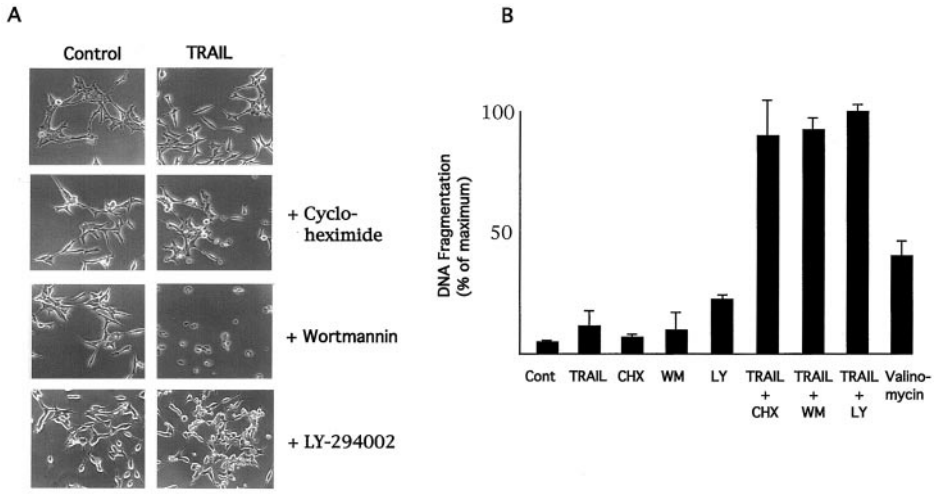

FIG. 3. **Inhibitors of PI 3-kinase or protein synthesis potentiate the cytotoxic activity of TRAIL.** *A*, LNCaP cells were treated for 24 h with 1 μg/ml TRAIL, 200 nM wortmannin, 20 μM LY-294002, or 10 μM cycloheximide alone or in combinations. The cells were visualized by light microscopy. *B*, LNCaP cells were treated for 6 h with 1 μg/ml TRAIL, 200 nM wortmannin (*WM*), 20 μM LY-294002, 10 μM cycloheximide (*CHX*), or 100 μM valinomycin alone or in combinations. DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone complexes released into the cytoplasm using a Cell Death Detection ELISA^{plus} kit.

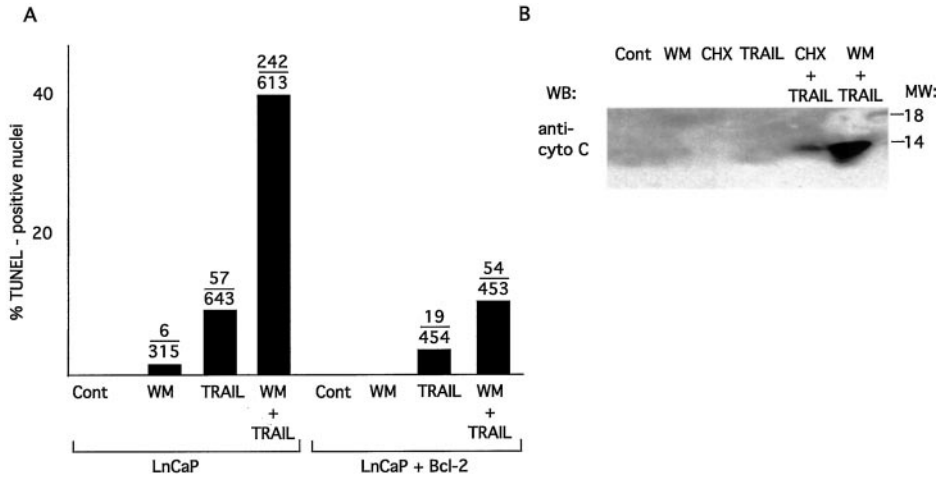

FIG. 4. **The role of mitochondrial cytochrome *c* release for TRAIL-induced apoptosis in LNCaP cells.** *A*, parental LNCaP cells or LNCaP cells overexpressing Bcl-2 were treated as described in the legend to Fig. 3*B*, and apoptotic nuclei were scored by TUNEL staining. Several randomly chosen microscopic fields were visualized, and both normal and TUNEL-positive cells were counted. The numbers of TUNEL-positive *versus* total numbers of counted cells are represented as ratios *above* the *bar graphs*. *B*, LNCaP cells were treated with TRAIL, wortmannin, or cycloheximide as described above. Cells were lysed in hypotonic buffer, and cytochrome *c* in the cytosolic fraction was measured by immunoblotting with cytochrome *c*-specific antibodies.

TRAIL is combined with cycloheximide and wortmannin (Fig. 5*A*). Similarly, these two compounds did not enhance TRAIL-induced cleavage of the apoptosis inhibitory protein XIAP, a substrate for several caspases including caspase 8 (51). These results suggest that the antiapoptotic block in LNCaP occurs downstream of caspase 8 activation. In contrast, proteolytic cleavage of the caspase 8 substrate BID was not detected in TRAIL-treated cells unless TRAIL was administered in combination with cycloheximide or wortmannin. Caspase 8-mediated cleavage of BID generates a proteolytic fragment, tBID, that is capable of inducing mitochondrial cytochrome *c* release and providing a functional link between death receptors and the mitochondria (28, 29). The lack of BID cleavage is thus consistent with the observation that TRAIL alone is not capable of inducing cytochrome *c* release. TRAIL-mediated processing of cytochrome *c*-dependent caspase 9 and effector caspase 7 were also detected only if TRAIL was combined with wortmannin or cycloheximide. The involvement of PI 3-kinase in the blockage of TRAIL-induced BID cleavage was further confirmed by the experiment with another PI 3-kinase inhibitor, LY-294002. Fig. 5*B* demonstrates that treatment of LNCaP cells with LY-294002 in combination with TRAIL results in the decreasing of cellular BID level. Thus, the PI 3-kinase- and protein synthesis-dependent antiapoptotic block in LNCaP cells occurs downstream of caspase 8, at the level of BID cleavage.

Alternatively, it is possible that the lack of BID cleavage may result from an inhibition of mitochondrial function. By analogy with the CD95/Fas system, LNCaP cells may be classified as type II cells, since mitochondrial function appears to be necessary for apoptosis. In type II cells, mitochondrial cytochrome *c* release serves as an amplification loop that potentiates the activation of caspase 8. If a similar mitochondria-dependent amplification loop is involved in TRAIL signaling in LNCaP cells, its disruption may affect caspase 8-mediated BID cleavage. To test whether or not cleavage of BID in LNCaP cells depends on mitochondrial function, the processing of BID in Bcl-2 overexpressor LNCaP cells *versus* parental cells was examined. Immunoblot analysis (Fig. 5*C*) demonstrates that after 6 h of treatment with TRAIL plus wortmannin or TRAIL plus cycloheximide, BID is processed equally well in parental and Bcl-2-overexpressing LNCaP cells. In addition, caspase 8 was processed efficiently in both cell lines as judged by the TRAIL-induced appearance of a cleavage product that corresponds to the 20-kDa active subunit of caspase 8. Thus, apoptogenic activity of mitochondria is not required for TRAIL-induced cleavage of BID and caspase 8.

Our results demonstrate that the blockage of TRAIL-induced apoptosis at the level of BID cleavage can be removed by cycloheximide treatment, suggesting the possibility that this inhibition may be mediated by a short lived protein. It has been hypothesized that inhibition of protein synthesis sensitizes cells to death-inducing ligands by down-regulating antiapoptotic cFLIP proteins (15, 19, 52). To determine whether this is the case for LNCaP cells, cell lysates from a previous experiment (Fig. 5*A*) were immunoblotted with antibodies that recognize different splice variants of cFLIP proteins: $FLIP_L$, FLIPγ, and FLIPδ (53). In contrast to published data, treatment of LNCaP cells for up to 16 h with cycloheximide or wortmannin had no effect on the level of cFLIP proteins (Fig. 5*C*), suggesting that they are unlikely to be involved in the

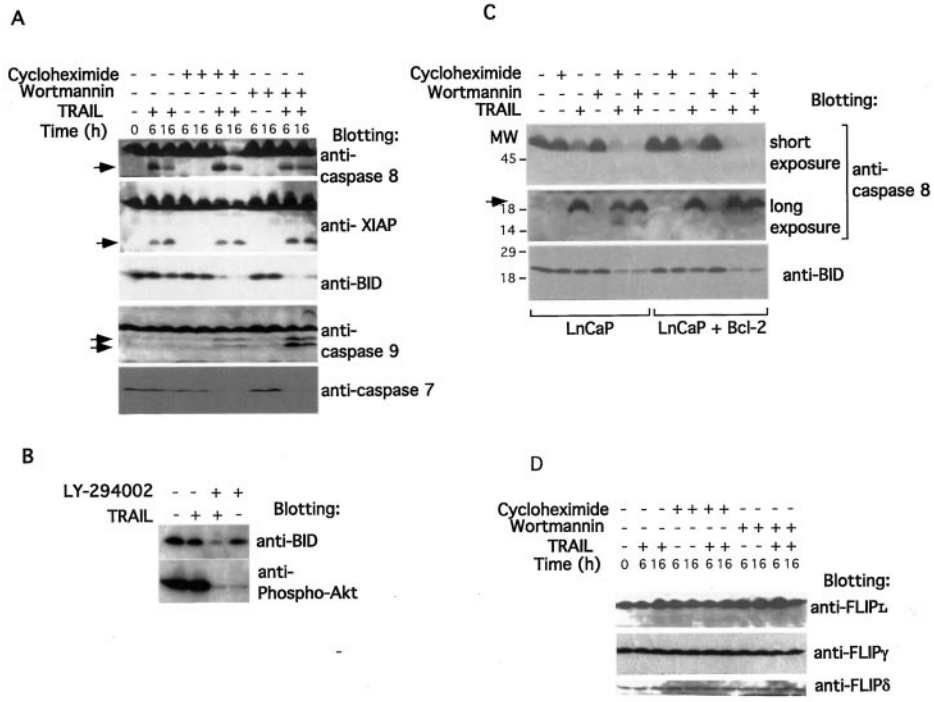

FIG. 5. **Block of TRAIL-mediated apoptotic signal in LNCaP cells occurs at the level of BID cleavage.** *A*, LNCaP cells were treated for 6 or 16 h with 1 μg/ml TRAIL, 200 nM wortmannin (*WM*), or 10 μM cycloheximide (*CHX*) alone or in combinations. Cell lysates were electrophoresed and consecutively immunoblotted with antibodies specific to caspase 8, XIAP, BID, caspase 9, and caspase 7. The *arrows* on the *left* indicate cleavage products. *B*, LNCaP cells were treated for 6 h with 1 μg/ml TRAIL or 20 μM LY-294002 alone or in combinations. Cell lysates were electrophoresed and consecutively immunoblotted with antibodies specific to BID or the phosphorylated form of Akt (Ser^{473}). *C*, parental LNCaP cells and LNCaP cells overexpressing Bcl-2 were treated for 6 h with 1 μg/ml TRAIL and 200 nM wortmannin alone or in combination. Cleavage of caspase 8 and BID was analyzed by immunoblotting with the corresponding antibodies. Blots were processed by ECL, and two different exposures were taken to visualize holocaspase 8 (short exposure) and its 20-kDa proteolytic fragment (long exposure). The *arrow* indicates caspase 8 cleavage product. *D*, cell lysates from the experiment described for *A* were immunoblotted with antibodies that specifically recognize different splice variants of cFLIP protein: $FLIP_L$, FLIPγ, and FLIPδ.

inhibition of TRAIL signaling in LNCaP cells.

Constitutively Active Akt Blocks TRAIL/Wortmannin-induced BID Cleavage—The potentiating effect of wortmannin on TRAIL-induced BID cleavage suggests that Akt may be involved in the inhibition of TRAIL signaling in LNCaP cells. To confirm this hypothesis, a constitutively active Akt, constructed by fusing Akt to the myristoylation signal of Src protein (myr-Akt) was introduced into LNCaP cells by adenovirus-mediated gene transfer. If Akt is the sole target of the wortmannin effect, then this infection would be expected to counteract the ability of wortmannin to sensitize LNCaP cells to TRAIL-induced BID cleavage. As a control, an adenovirus containing kinase-inactive Akt (myr-Akt(K−) was used. LNCaP cells infected with adenoviral constructs 16 h prior to the experiment were treated for an additional 6 h with TRAIL or TRAIL plus wortmannin, and BID cleavage was examined by immunoblotting. Our results demonstrate (Fig. 6*A*) that the infection of LNCaP cells with myr-Akt, but not with the kinase-inactive Akt, inhibits processing of BID induced by TRAIL plus wortmannin treatment. TRAIL-mediated cell death was also inhibited in myr-Akt-infected cells as judged by cell morphology (data not shown). Thus, activated Akt is capable of rescuing LNCaP cells from the apoptogenic action of TRAIL plus wortmannin treatment, supporting the hypothesis that the resistance of LNCaP cells to TRAIL results from high constitutive activity of Akt.

We next tested whether activated Akt can also inhibit cleavage of BID induced by TRAIL plus cycloheximide treatment. However, no rescue was observed even when the adenovirus titer was 16 times higher than that sufficient to inhibit pro-apoptotic effects of TRAIL plus wortmannin treatment (Fig. 6*B*). These results suggest that the protective effects of Akt on BID cleavage may require Akt-induced protein synthesis.

Our results (Figs. 1*B* and 2*A*) indicate the existence of TRAIL-sensitive cell lines that possess an elevated Akt activity, albeit at a much lower level than that found in LNCaP cells. This result raises the question of whether the protective effect of Akt is cell type-specific or it occurs only when the level of Akt activity is above a certain threshold. To examine these possibilities, we overexpressed myristoylated Akt in various TRAIL-sensitive cell lines: DU 145 and ALVA-31 prostate cancer cells, A498 renal cancer cells, and HeLa cervical cancer cells. Of them, only ALVA-31 cells acquired significant resistance to TRAIL upon myr-Akt overexpression (Fig. 6*C*). Thus, the protective effect of Akt appears to be cell type-specific.

DISCUSSION

We have developed a novel approach to obtaining preparative amounts of proapoptotic ligand TRAIL and tested the effects of this reagent on a panel of six prostate cancer cell lines. Soluble TRAIL was produced by a methylotrophic yeast *P. pastoris*, secreted into the medium, and then purified to homogeneity by one-step chromatography on a nickel-chelate column. Cytotoxicity assays demonstrated that three cell lines, ALVA-31, DU 145, and PC-3, were very sensitive to TRAIL, while in comparison JCA-1 and TSU-Pr1 revealed moderate sensitivity, and LNCaP cells were resistant to as high as 4 μg/ml TRAIL. Comparing these results with the data published on Fas ligand-induced apoptosis indicates that prostate cancer cells differ in their responses to these two apoptotic stimuli. Whereas cells believed to be derived from primary prostate cancer tumors (ALVA-31 and JCA-1) were reported to be sensitive to Fas ligand-induced apoptosis, cells originating from distant metastasis (DU 145, PC-3, TSU-Pr1, and LNCaP) appeared to be Fas-resistant despite the expression of Fas antigen on the cell surface (36, 54). In contrast, among the above listed cell lines, only LNCaP cells were resistant to TRAIL-induced apoptosis, indicating that TRAIL has a greater potential as an agent to treat metastatic prostate cancer. These data also suggest that despite the similarity of CD95/Fas and TRAIL receptors, TRAIL and Fas ligand-mediated apoptosis may employ different signal transduction pathways or be negatively regulated by different mechanisms in these prostate cancer cells.

We found that among six prostate cancer cell lines examined, the LNCaP cells, which are the most highly resistant to TRAIL-induced apoptosis, have the highest constitutive activity of the Akt protein kinase. This result is consistent with the lack of the functional tumor suppressor PTEN, a negative regulator of the PI 3-kinase/Akt pathway in these cells (42). Because the Akt

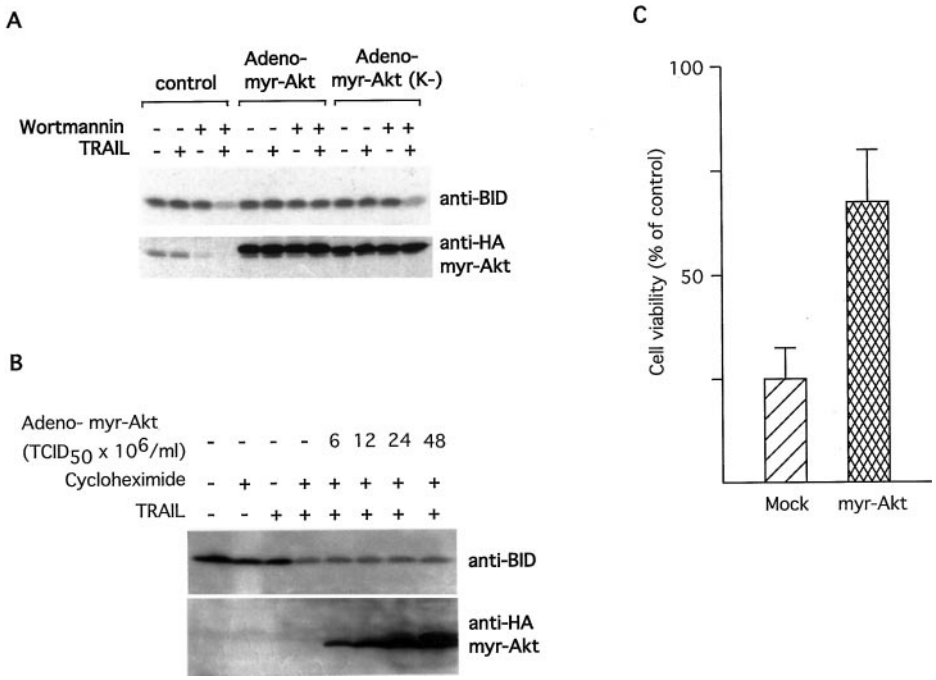

FIG. 6. **Constitutively active Akt inhibits proapoptotic effects of TRAIL.** *A*, LNCaP cells were infected with adenoviral constructs expressing myristoylated Akt (*Adeno-myr-Akt*) or kinase-inactive myristoylated Akt (*Adeno-myr-Akt(K−)*) at a titer of 3×10^6 $TCID_{50}$/ml. Control cells were not infected with adenoviruses. 16 h postinfection, the cells were treated for 6 h with 1 μg/ml TRAIL and 200 nM wortmannin alone or in combination. Cell lysates were consecutively probed with BID-specific antibody and anti-HA1 antibody that recognizes hemagglutinin-tagged myr-Akt. *B*, LNCaP cells were infected where indicated with adenoviral constructs expressing myristoylated Akt (*Adeno-myr-Akt*) at a titer increasing from 6 to 48 $\times$ 10^6 $TCID_{50}$/ml. Control cells were not infected with adenovirus. 16 h after infection, the cells were treated for 6 h with 1 μg/ml of TRAIL and 10 μM cycloheximide alone or in combination. Cell lysates were probed as outlined for *A*. *C*, ALVA-31 cells were transiently cotransfected with an expression plasmid encoding the *E. coli lacZ* gene plus an expression plasmid for myristoylated Akt (*myr-Akt*) (60) or empty expression vector (*Mock*). 24 h after transfection, the cells were incubated with or without 0.1 μg/ml TRAIL and scored for apoptosis 24 h later. Cells positive for β-galactosidase activity were checked for morphological changes characteristic of apoptosis, and the percentage of live cells was quantitated.

protein kinase is known to block apoptosis (55), we tested whether inhibition of this pathway affects the sensitivity of LNCaP cells to TRAIL. We found that treatment with the PI 3-kinase inhibitors wortmannin and LY-294002 or the protein synthesis inhibitor cycloheximide renders them sensitive to TRAIL-induced apoptosis. Thus, the resistance of LNCaP cells to TRAIL results not from defects in apoptotic machinery, but from PI 3-kinase-dependent inhibition of the TRAIL-mediated apoptotic signaling pathway.

It has been reported that apoptosis induced by triggering of CD95/Fas (56, 57) is counteracted by the PI 3-kinase/Akt pathway, but the molecular mechanisms that cause apoptosis resistance remain unclear. To identify which step of the TRAIL-mediated apoptotic pathway is blocked in LNCaP cells, we first tested whether the release of proapoptotic factors from mitochondria is essential for TRAIL-induced death of these cells. The involvement of mitochondria in apoptosis induced by death receptors remains controversial. Scaffidi *et al.* (30) have proposed that two types of cells exist that differ with respect to their requirement for mitochondria during Fas-mediated apoptosis. In type I cells, caspase 8 is activated without involvement of mitochondria to a level sufficient to process the effector caspase 3. In contrast, in type II cells a mitochondria-dependent amplification loop is required to fully activate caspase 8 and transduce an apoptotic signal. This model has recently been questioned by Huang *et al*. (58), who argue that the difference between type I and type II cells is an artifact of using agonistic anti-Fas antibodies to trigger Fas signaling instead of Fas ligand. To clarify the role of mitochondria in TRAIL-induced apoptosis in LNCaP cells, we used Bcl-2-overexpressing LNCaP cells, which were shown to exhibit an impaired cytochrome *c* release in response to various apoptotic stimuli (37). Our results demonstrate that these cells are much more resistant to TRAIL plus wortmannin-induced apoptosis compared with the parental cells. In these experiments, apoptosis was triggered by soluble death receptor ligand and not agonistic antibody, supporting the notion that in some cells mitochondrial function is indeed essential for death receptor-mediated apoptosis.

Using a cell fractionation approach, we have found that TRAIL-induced cytochrome *c* release was blocked in LNCaP cells, but both wortmannin and cycloheximide are capable of overcoming this block. Release of mitochondrial cytochrome *c* by death receptors is triggered by a multistep mechanism. The formation of the DISC results in autoprocessing and activation of the initiator caspase 8 followed by cleavage of the proapoptotic protein BID (28, 29). A proteolytic fragment of BID translocates to the mitochondria as an integral membrane protein and triggers the release of mitochondrial cytochrome *c* (59). Using immunoblot analysis, we found that cleavage of caspase 8 and one of its substrates, the antiapoptotic protein XIAP (51) were induced by TRAIL alone as efficiently as when TRAIL was combined with either wortmannin or cycloheximide. This important result indicates that DISC formation or caspase 8 activation was not blocked in LNCaP cells. In contrast, wortmannin and cycloheximide were required for TRAIL-induced cleavage of BID, the release of cytochrome *c*, and processing of caspases 9 and 7. Thus, the PI 3-kinase-dependent block of TRAIL-induced apoptosis in LNCaP cells occurs at the level of BID cleavage.

The requirement for mitochondrial apoptogenic activity in TRAIL-induced death suggests that LNCaP cells are similar to type II cells. If so, the lack of BID cleavage could, in principle, be explained by the disruption of a mitochondria-dependent

amplification loop, resulting in only partial activation of caspase 8. To see whether this hypothesis could be true, we compared the cleavage of BID and caspase 8 in Bcl-2-overexpressing *versus* parental LNCaP cells and found that these proteins are processed equally well in both cell lines. These results demonstrate that although mitochondrial function is important for TRAIL-induced apoptosis in LNCaP cells, unlike "typical" type II cells mitochondria are required not to amplify caspase 8 activation but to transduce apoptotic signal downstream of the initiator caspase. Therefore, it may be possible to classify LNCaP as type III cells where mitochondria are involved in the propagation rather than the initiation of the apoptotic cascade.

Involvement of PI 3-kinase in the block of apoptosis suggests that Akt could mediate resistance of LNCaP cells to TRAIL. To confirm this hypothesis, we tested whether overexpression of constitutively active Akt could inhibit the proapoptotic effect of TRAIL plus wortmannin treatment. For this purpose, we used a myristoylated derivative of Akt, which exhibits kinase activity independently of PI 3-kinase (60). Both apoptosis (data not shown) and BID cleavage induced by treatment of LNCaP cells with TRAIL plus wortmannin were inhibited by overexpression of myristoylated Akt, indicating that resistance of LNCaP cells to TRAIL is, at least in part, mediated by Akt.

It has been documented that Akt may inhibit a variety of apoptotic stimuli in multiple ways (55). These include direct phosphorylation and modulation of proapoptotic proteins BAD (48) and caspase 9 (50), activation of antiapoptotic NF-κB-mediated transcriptional pathways (61, 62), or phosphorylation of the Forkhead family of transcription factors, preventing them from inducing the transcription of proapoptotic genes (63). Inhibition of BID cleavage has not been previously reported as a mechanism through which PI 3-kinase and Akt block apoptotic signals.

Although it remains unclear how the PI 3-kinase/Akt pathway mediates inhibition of BID cleavage, our data suggest an indirect mechanism. First, inhibition of protein synthesis by cycloheximide affected the same step of TRAIL apoptotic cascade as the inhibition of PI 3-kinase. However, even very high levels of constitutively active Akt did not rescue BID from cleavage when TRAIL was combined with cycloheximide rather than wortmannin. These results suggest that a short lived protein is involved in the PI 3-kinase/Akt-mediated blockage of BID cleavage, and the synthesis of this hypothetical protein may be triggered by Akt. Second, the effect of myristoylated Akt appears to be cell type-specific, since its overexpression did not rescue HeLa, DU-145, or A498 cells from TRAIL-induced apoptosis (data not shown) but did rescue LNCaP and ALVA-31 cells. This could reflect either the difference in apoptotic pathways employed by different cell types or the absence of certain factors required for the protective effect of Akt. In particular, human prostate cancer cell lines have scores of chromosomal deletions and rearrangements (64), so that LNCaP and PC-3 differ in much more than Akt levels.

It has been reported that short term (3–7-h) treatment of human keratinocytes (52), HeLa and Kym-1 cells (19) with cycloheximide significantly reduces the level of cellular cFLIP protein. Since upon overexpression cFLIP is capable of inhibiting Fas-mediated apoptosis (33, 34), it has been suggested that protein synthesis inhibitors sensitize cells to TRAIL by down-regulating cFLIP. To examine this hypothesis, we tested the level of various splice variants of cFLIP ($FLIP_L$, FLIPγ, and FLIPδ) in LNCaP cells and found that neither cycloheximide nor wortmannin treatment affected cFLIP levels after as long as 16 h of treatment. These data are consistent with our observation on renal carcinoma cells (65) and published results on Kaposi's sarcoma cells (17) in which that inhibition of protein synthesis sensitized cells to TRAIL without affecting the expression of cFLIP proteins. Thus, mediators of the PI 3-kinase-dependent blockage of TRAIL-induced BID cleavage and apoptosis in LNCaP cells still await identification and characterization.

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Oncogenic Ras Sensitizes Normal Human Cells to Tumor Necrosis Factor-α-Related Apoptosis-Inducing Ligand-Induced Apoptosis

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ABSTRACT

Tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL) is a cytotoxic cytokine that induces apoptosis in tumor cells but rarely kills normal ones. To determine how normal human cells acquire TRAIL-sensitive phenotype during the process of malignant transformation, we used an experimental system that allows for controlled conversion of human cells from normal to cancerous by introduction of several genes. Human embryonic kidney cells and foreskin fibroblasts were first immortalized by combination of the early region of simian virus 40 and telomerase and then were transformed with oncogenic Ras. Both normal and immortalized cells were resistant to TRAIL-induced apoptosis, whereas Ras-transformed cells were susceptible. Ras transformation enhanced TRAIL-induced activation of caspase 8 by increasing its recruitment to TRAIL receptors. The proapoptotic effects of Ras could be reversed by mutations in its effector loop or by inhibitors of either farnesyl transferase or mitogen-activated protein kinase kinase. The expression of constitutively activated mitogen-activated protein kinase kinase 1 enhanced caspase 8 recruitment and sensitized immortalized human embryonic kidney cells to TRAIL-induced death. These results indicate that in normal human cells the TRAIL-induced apoptotic signal is blocked at the level of caspase 8 recruitment and that this block can be eliminated by Ras transformation, involving activation of the mitogen-activated protein kinase pathway.

INTRODUCTION

Tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL) is a cytotoxic cytokine that selectively induces apoptosis in a variety of tumor cells but rarely affects normal ones (1). At least five receptors for TRAIL have been identified. Two of these, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), contain a conserved motif, the death domain, and signal apoptosis (2). Binding of TRAIL to DR4 or DR5 induces formation of the death-inducing signaling complex (DISC), which comprises the adaptor protein Fas-associated death domain (FADD)/MORT1 and the FADD-binding cysteine protease, caspase 8/FLICE (3, 4). The formation of the DISC triggers proteolytic autoprocessing and activation of caspase 8, which in turn cleaves and activates the downstream caspases 3, 6, or 7, leading to apoptosis. In addition, caspase 8 cleaves a cytolinker, plectin, which induces reorganization of the cytoskeleton (5), and the proapoptotic protein, Bid, which generates a cleavage product that triggers the release of cytochrome *c* from mitochondria (6, 7).

The reason that TRAIL is selective toward cancer cells despite the ubiquitous expression of its receptors in normal tissues remains unclear. Malignant transformation is believed to require stepwise accumulation of at least three genetic alterations: inactivation of tumor suppressors, immortalization, and receipt of a continuous mitogenic signal (8). To investigate at which step of this process and how human cells acquire a TRAIL-sensitive phenotype, we used a recently developed experimental system that mimics stepwise progression of human cells from normal to tumorigenic (9). Normal cells were converted to tumorigenic by serial introduction of the early region of simian virus (SV40ER) to disable tumor suppressors p53 and Rb, a catalytic subunit of telomerase (hTERT) to ensure their unlimited life span, and an oncogenic allele of Ha-Ras (H-*ras*-V12) to provide cells with a continuous growth signal. On transformation with activated Ras, these cells become capable of anchorage-independent growth and formation of tumors in immunodeficient mice (9). This model allowed us to investigate a mechanism by which malignant transformation renders human cells susceptible to TRAIL-induced apoptosis.

MATERIALS AND METHODS

Reagents. The following reagents were obtained from the indicated sources: SCH 66336 (Schering-Plough Research Institute); PD 98059 and U0126 (Calbiochem); z-VAD-FMK (Enzyme System Products); anti-phospho-mitogen-activated protein kinase (anti-phospho-ERK) and anti-ERK (New England Biolabs); anti-Bid (Zymed Laboratories); antiplectin (Transduction Laboratories); anti-caspase 8 and anti-FADD (Upstate Biotechnology); monoclonal antibody to caspase 10 (Clone 4C1; MBL International); anti-DR4 and anti-DR5 (Alexis); monoclonal antibody NF6 to FLIP (a gift of Marcus Peter, Ben May Institute for Cancer Research). The expression and purification of TRAIL from yeast *Pichia pastoris* has been described in detail elsewhere (10).

Cell Culture. Normal human foreskin fibroblasts (BJ) were obtained from the American Type Culture Collection. Normal human embryonic kidney (HEK) cells were kindly provided by Silvia Bacchetti (McMaster University). Immortalization of the cells with SV40 large T antigen and hTERT and their subsequent transformation with H-*ras*-V12 has been described in detail elsewhere (9). Different Ras and mitogen-activated protein kinase kinase (MEK) constructs were expressed in immortalized HEK cells by use of pBabe-Puro vector (Ref. 11; a gift of Scott W. Lowe, Cold Spring Harbor Laboratory). Retroviral stocks were generated in Phoenix ecotropic packaging line (G. Nolan, Stanford University), and stable transformants were selected in the presence of puromycin (500 ng/ml). Cells infected with an empty vector were used as a control. Human bladder cancer cell line T24 was kindly provided by Gary J. Miller (University of Colorado Health Sciences Center, Denver, CO).

Cytotoxicity Assays. Cell viability was determined by either the tetrazolium-based Aqueous One assay (Promega) or by staining with 7-aminoactinomycin D and flow cytometry. Detection of DNA fragmentation by agarose gel electrophoresis was performed with a Suicide-Track DNA Ladder Isolation Kit (Oncogene Research Products), using a procedure that selectively extracts apoptotic DNA from intact chromatin.

DISC Immunoprecipitation. HEK cells grown in roller bottles ($\sim 4 \times 10^8$ cells/condition) were scraped into 10 ml of conditioned medium, combined, precipitated, and resuspended in 5 ml of the conditioned medium. As judged by the trypan blue exclusion assay, >80% of cells remained viable after this procedure. Stimulation with TRAIL was achieved by incubation with TRAIL (1 μg/ml) for 20 min at 37°C. The cells were washed in ice-cold PBS, lysed in 10 ml of Triton/glycerol/HEPES buffer (12), cleared by centrifugation, and equalized for protein content. Precipitation of the unstimulated TRAIL receptors was achieved by adding TRAIL (1 μg/ml) to the lysates for 30 min at 4°C. The TRAIL receptors that were complexed with TRAIL were immunoprecipitated by addition of 25 μl of antipolyhistidine agarose (Sigma) for 2 h at 4°C, and bound proteins were eluted with 100 mM glycine-HCl (pH 2.3; two times 40 μl for 10 min at 4°C).

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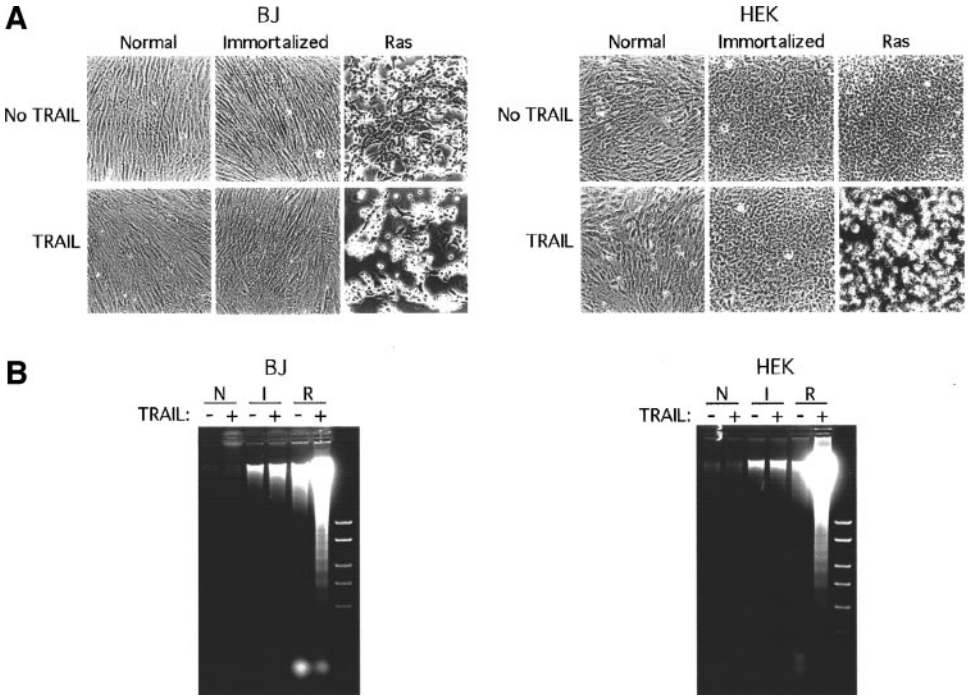

Fig. 1. Transformation of human embryonic kidney cells (*HEK*) and human foreskin fibroblasts (*BJ*) with Ras sensitizes them to tumor necrosis factor-α-related apoptosis-inducing ligand (*TRAIL*)-induced apoptosis. The effects of TRAIL (1 μg/ml) on normal (*N*), immortalized (*I*), or Ras-transformed (*R*) HEK cells and BJ fibroblasts were assessed 72 h after the addition of TRAIL. *A*, the morphology of the plated cells was assessed, and representative photographs are shown. *B*, the cells were harvested, and extracts were processed by DNA laddering assay. DNA laddering was visualized by ethidium bromide staining of 2% agarose gels. Molecular weight markers are shown on the *right*.

RESULTS

Conversion of Normal Human Cells to Tumorigenic Ones Sensitizes Them to TRAIL. HEK and BJ cells were first immortalized by the combination of SV40ER and hTERT and then transformed with oncogenic Ras (9). Both normal and immortalized cells remained resistant to TRAIL, whereas the introduction of activated Ras rendered them susceptible to TRAIL-induced apoptosis, as assessed by both morphological changes typical of apoptosis (Fig. 1*A*) and DNA fragmentation (Fig. 1*B*).

Ras Transformation Potentiates TRAIL-Induced Activation of the Initiator Caspase 8. Because the process of immortalization did not sensitize cells to TRAIL-induced apoptosis, these cells were used as the control for additional experiments. To determine what step in the TRAIL-mediated apoptotic cascade is enhanced by Ras transformation, we compared TRAIL-induced proteolytic events in Ras-transformed and control, immortalized cells. As shown in Fig. 2, the addition of TRAIL to control cells did not induce cleavage of the initiator caspase 8. In contrast, treatment of Ras-transformed cells with TRAIL induced significant caspase 8 cleavage. Interestingly, TRAIL-induced processing of another proximal caspase, caspase 10 (13), was not facilitated by Ras transformation (Fig. 2), suggesting that these two enzymes may be regulated in different ways.

Caspase 8 cleavage is not necessarily accompanied by an increase in the activity of this proteolytic enzyme toward its cellular substrates (14). We therefore examined the ability of Ras to enhance TRAIL-induced cleavage of two specific caspase 8 substrates, Bid (6, 7) and plectin (5). As shown in Fig. 2, oncogenic Ras enhanced the ability of TRAIL to induce the cleavage of plectin in both HEK and BJ cells. Although we could not detect significant cleavage of Bid in BJ cells, proteolytic processing of this protein was evident in Ras-transformed HEK cells, which were generally more responsive to TRAIL than BJ fibroblasts.

Ras Transformation Enhances Recruitment of Caspase 8 to TRAIL DISC. We next examined how transformation of cells with Ras affected the ability of TRAIL receptors to form a functional DISC. To immunoprecipitate the activated TRAIL receptors DR4 and DR5, we first incubated intact cells with a $(His)_6$-tagged recombinant TRAIL (10) and then immunoprecipitated the receptors with an anti-polyhistidine antibody. To immunoprecipitate unstimulated receptors, we first lysed cells with detergent and then added $(His)_6$-TRAIL to the extracts.

These and subsequent experiments were performed with the HEK cells because the proapoptotic effects of Ras transformation were more robust in these cells. As shown in Fig. 3, the antibody to $(His)_6$-TRAIL efficiently immunoprecipitated both TRAIL receptors, DR4 and DR5. The lack of immunoprecipitation of TRAIL receptors when the cells or cell lysates were not treated with $(His)_6$-TRAIL

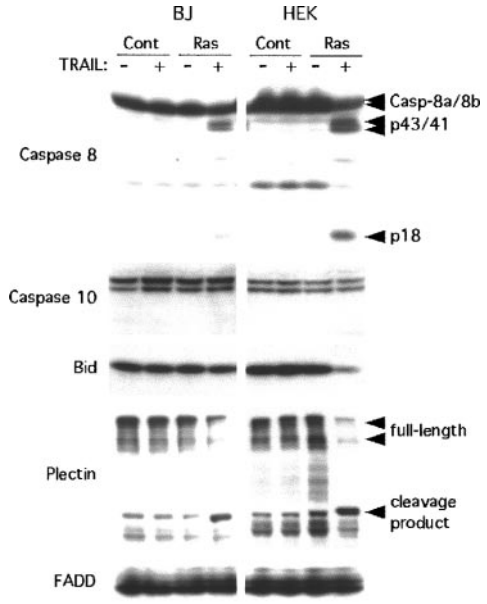

Fig. 2. Transformation of cells with Ras enhances the ability of tumor necrosis factor-α-related apoptosis-inducing ligand (*TRAIL*) to activate caspase 8. Control (*Cont*) cells (immortalized cells infected with empty vector) or Ras-transformed human embryonic kidney cells (*HEK*) and human foreskin fibroblasts (*BJ*) were treated for 72 h with 1 μg/ml TRAIL. Cell lysates were immunoblotted with antibodies specific for caspase 8, caspase 10, Bid, and plectin. Equal protein loading was confirmed by probing the blot with antibodies to Fas-associated death domain (*FADD*).

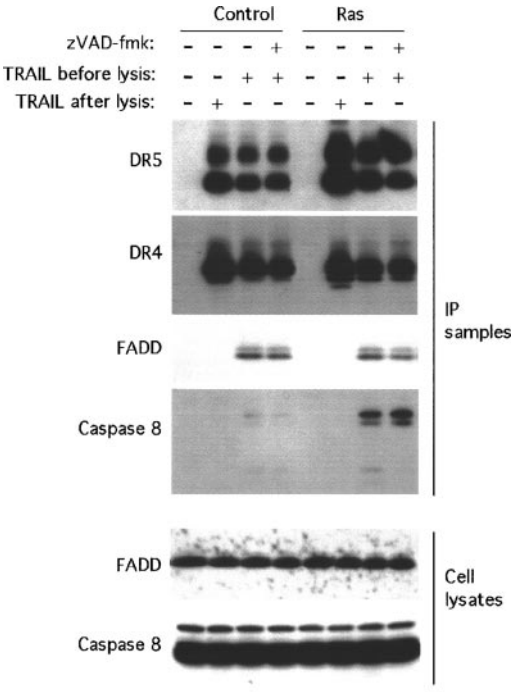

Fig. 3. Transformation of cells with Ras enhances the recruitment of caspase 8 to Fas-associated death domain (*FADD*). Control and Ras-transformed human embryonic kidney cells (HEK) were treated with $(His)_6$-tagged tumor necrosis factor-α-related apoptosis-inducing ligand (1 μg/ml) for 20 min at 37°C (*TRAIL before lysis*). The cells were lysed, and TRAIL–TRAIL-receptor complexes were immunoprecipitated (*IP*) with monoclonal antibody to polyhistidine. To immunoprecipitate unstimulated receptors, TRAIL was added to cells after lysis. Aliquots of the immunoprecipitated material were analyzed by immunoblotting with antibodies specific for DR5, DR4, FADD, and caspase 8. DR5 is detected as a doublet, corresponding to the two different splice variants of this protein (15). Equal amounts of protein in cell lysates was confirmed by probing with antibodies to FADD and caspase 8.

confirmed the specificity of this procedure. DR5 was detected as a doublet, corresponding to two known splice variants of this protein (15). This result demonstrates that oncogenic Ras does not significantly affect the level of DR4 in these cells but elevates the expression of DR5. Densitometric analysis of two independent experiments revealed the 1.5–2-fold increase of DR5 expression in Ras-transformed *versus* control cells.

When cells were treated with TRAIL before lysis, two additional proteins, FADD and caspase 8, coimmunoprecipitated with the TRAIL receptors (Fig. 3). FADD coimmunoprecipitated equivalently with TRAIL receptors from Ras-transformed and control cells. In contrast, caspase 8 was recruited to TRAIL receptors in Ras-transformed cells much more efficiently than in control cells. Densitometric analysis of two independent experiments demonstrated that Ras increased caspase 8 recruitment ≥8-fold. This result suggests that Ras transformation sensitizes cells to TRAIL by facilitating the binding of caspase 8 to FADD.

To rule out the possibility that the observed difference in the amounts of coimmunoprecipitated caspase 8 resulted from different rates of caspase 8 processing and subsequent dissociation from the DISC, cells were pretreated with the pancaspase inhibitor Z-VAD-FMK. Inhibition of caspase 8 activity, however, did not have a significant effect on the amounts of caspase 8 bound to the TRAIL receptors (Fig. 3).

The Proapoptotic Effects of Ras Are Reversible. To test whether sensitization of cells to TRAIL by Ras transformation is a reversible process, we used the farnesyl transferase inhibitor SCH 66336, a compound that inhibits prenylation of Ras proteins and suppresses their biological activity (16). As shown in Fig. 4, *A* and *B*, pretreatment of Ras-transformed HEK cells with SCH 66336 efficiently rescued them from TRAIL-induced death.

To test whether the Ras-induced MAP kinase pathway contributes to the proapoptotic effect of this oncogene, we used two inhibitors of MEK, PD 98059 (17) and U0126 (18). As shown in Fig. 4, *A* and *B*, the MEK inhibitors efficiently rescued Ras-transformed HEK cells from TRAIL. Both the MEK and farnesyl transferase inhibitors suppressed TRAIL-induced cleavage of caspase 8 (Fig. 4*C*). These compounds thus appear to specifically reverse the proapoptotic effects of Ras.

We next tested whether Ras-dependent sensitization of cells to TRAIL-induced apoptosis can also occur in transformed cells obtained from cancer patients. For this purpose we used the bladder cancer cell line T24, which expresses the oncogenic allele of Ha-Ras (H-*ras*-V12) and possesses constitutively activated MAP kinase (19).

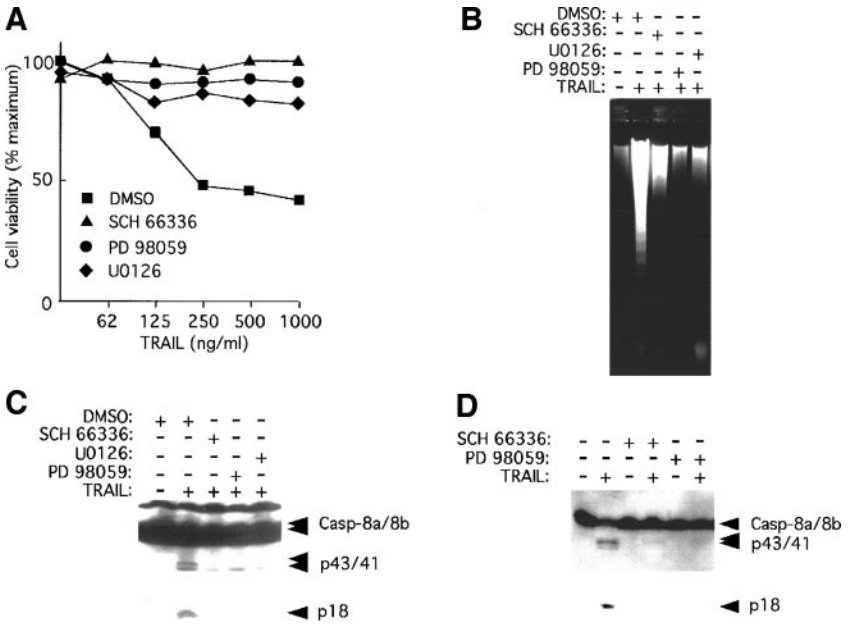

Fig. 4. Effects of small molecule inhibitors on tumor necrosis factor-α-related apoptosis-inducing ligand (*TRAIL*)-induced apoptosis of Ras-transformed cells. *A* and *B*, Ras-transformed human embryonic kidney (HEK) cells were pretreated for 72 h with farnesyl transferase inhibitor SCH 66336 (1 μM), mitogen-activated protein (MEK) inhibitors PD 98059 (100 μM) and U0126 (20 μM), or 0.1% DMSO as a control. Cells were then treated for an additional 72 h with different concentrations of TRAIL. *A*, the relative viability of cells was evaluated by use of the tetrazolium conversion assay. *B*, the cells were harvested, and extracts were processed by DNA laddering assay. DNA laddering was visualized by ethidium bromide staining of 2% agarose gels. *C*, Ras-transformed HEK cells were pretreated with one of the small molecule inhibitors as described above and then incubated with TRAIL (1 μg/ml) for 48 h. After this treatment, the cells were lysed and immunoblotted with an antibody to caspase 8. A short exposure of the electrochemiluminescence-treated blot was used to visualize holocaspase 8, and a long exposure was used for its 18-kDa proteolytic fragment. *D*, T24 human bladder cancer cells were pretreated for 72 h with farnesyl transferase inhibitor SCH 66336 (1 μM), MEK inhibitor PD 98059 (100 μM), or 0.1% DMSO as a control. Where indicated, cells were then treated for an additional 24 h with TRAIL (1 μg/ml). After this treatment, the cells were lysed and immunoblotted with an antibody to caspase 8.

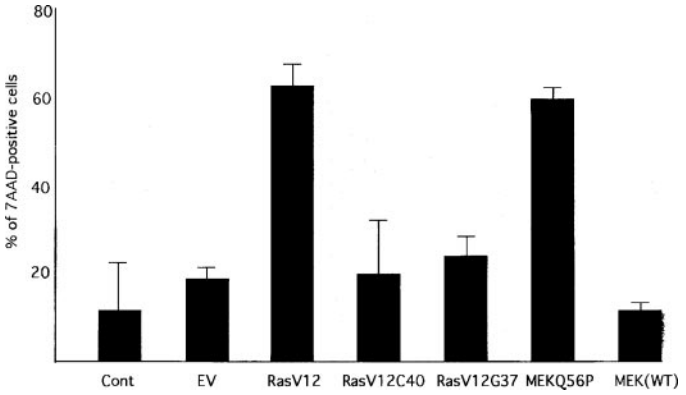

Fig. 5. Proapoptotic effects of Ras are mediated by the mitogen-activated protein kinase pathway. Immortalized human embryonic kidney (HEK) cells were infected with empty retrovirus (*EV*), retroviruses expressing different Ras constructs (as indicated), activated mutant of mitogen-activated protein kinase 1 (*MEK1Q56P*) or wild-type MEK1 [*MEK(WT)*]. Uninfected cells were used as control (*Cont*). The cells were treated with 1 μg/ml tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL) for 48 h. Apoptotic cells were detected by incorporation of 7-aminoactinomycin D (*7AAD*). Data are presented as percentage of 7AAD-positive cells after subtraction of 7AAD uptake by cells not treated with TRAIL. The data shown are the average of three determinations and the SD (*bars*) of the mean.

Using the farnesyl transferase inhibitor SCH 66336 or the MEK inhibitor PD 98059, we found that inhibition of either Ras processing or MAP kinase activity suppressed TRAIL-induced caspase 8 cleavage in T24 cells (Fig. 4*D*) and rescued them from TRAIL-mediated apoptosis (data not shown). This result confirms that sensitization of cells to TRAIL by oncogenic Ras can indeed take place in spontaneous human cancers.

The Proapoptotic Effects of Ras Are Mediated by MAP Kinase Pathway. The results presented in Fig. 4 suggested that the proapoptotic effects of Ras may involve the MAP kinase pathway. To confirm this observation, we transformed immortalized HEK cells with retroviruses expressing either a gain-of-function mutant of MEK1 ($MEK1^{Q56P}$), a dual-specificity protein kinase that phosphorylates and activates MAP kinase (11, 20), or two effector loop mutants of Ras (RasV12C40 and Ras V12G37) that are defective for Raf binding and do not activate the MAP kinase pathway (21–23). As measured by incorporation of 7-aminoactinomycin D, TRAIL efficiently induced death in cells that expressed either RasV12 or $MEK1^{Q56P}$ but not in cells expressing constructs defective in MAP kinase activation (Fig. 5). These results confirm that an activated MAP kinase pathway is essential for sensitization of HEK cells to TRAIL-induced apoptosis.

Triggering of MAP Kinase Pathway Enhances TRAIL-Induced Activation of Caspase 8. The results presented in Figs. 2 and 3 suggest that oncogenic Ras sensitizes cells to TRAIL-inducing apoptosis by enhancing activation of caspase 8. To investigate the involvement of the MAP kinase pathway in this process, we used a constitutively activated mutant of MEK1, $MEK1^{Q56P}$. Using an antibody that specifically recognizes the phosphorylated, activated form of MAP kinase, we found that expression of $MEK1^{Q56P}$ induced phosphorylation of MAP kinase to approximately the same level as that observed in Ras-transformed cells (Fig. 6*A*).

As demonstrated in Fig. 6*B*, activation of the MAP kinase pathway potentiates the same step in the TRAIL-induced apoptotic cascade as oncogenic Ras. Treatment of either Ras-transformed or $MEK1^{Q56P}$-expressing, but not control, cells with TRAIL induced cleavage of both caspase 8 and two caspase 8 substrates, $cFLIP_L$ and Bid.

We also tested whether TRAIL-induced activation of caspase 8 can be enhanced by some stress stimuli known to trigger the MAP kinase pathway. For this purpose we treated HEK cells with UV light, a potent activator of the ERK pathway (24). As demonstrated in Fig. 6*C*, UV irradiation resulted in greatly increased caspase 8 processing in response to TRAIL.

The results presented in Fig. 3 suggested that oncogenic Ras sensitizes cells to TRAIL by enhancing the recruitment of caspase 8 to

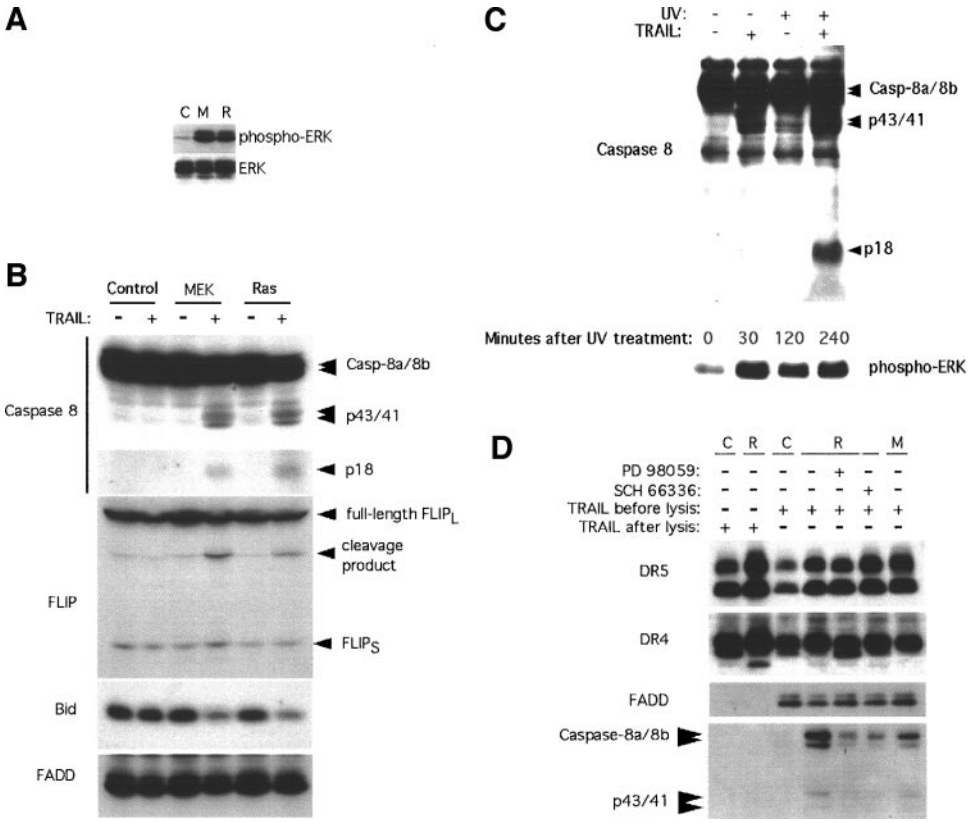

Fig. 6. Activation of the mitogen-activated protein (MAP) kinase pathway potentiates tumor necrosis factor-α-related apoptosis-inducing ligand (*TRAIL*)-induced recruitment of caspase 8. Human embryonic kidney (HEK) cells were infected by a retrovirus expressing constitutively the activated mutant of MAP kinase kinase (MEK), $MEK1^{Q56P}$. *A*, control cells (*C*), $MEK1^{Q56P}$-expressing cells (*M*), or Ras-transformed cells (*R*) were lysed and analyzed by immunoblotting with antibodies specific for phosphorylated MAP kinase (*phospho-ERK*) or total MAP kinase (*ERK*). *B*, control, $MEK1^{Q56P}$-expressing (*MEK*), or Ras-transformed cells were treated for 48 h with TRAIL (1 μg/ml). Cell lysates were immunoblotted with antibodies specific for caspase 8, FLIP, and Bid. Equal protein loading was confirmed by probing the blot with antibodies to Fas-associated death domain (*FADD*). *C*, *upper panel*, where indicated, immortalized HEK cells were exposed to UV irradiation at 100 $\mu J/cm^2$ and then treated with TRAIL (1 μg/ml) for 3 h. Cell lysates were subjected to Western blotting with an antibody to caspase-8. *Lower panel*, cells were irradiated as described above, followed by incubation for various times. Cell lysates were subjected to Western blotting with an antibody to phosphorylated ERK (*phospho-ERK*). *D*, TRAIL receptors were immunoprecipitated with the antibody to polyhistidine from control (*C*), Ras-transformed (*R*), or $MEK1^{Q56P}$-expressing cells (*M*) as described in the legend to Fig. 3*A*. Where indicated, cultures were pretreated for 72 h with farnesyl transferase inhibitor SCH 66336 (1 μM) or MEK inhibitor PD 98059 (100 μM). Aliquots of the immunoprecipitated materials were analyzed by immunoblotting with antibodies specific for DR4, DR5, caspase 8, and Fas-associated death domain (*FADD*).

TRAIL DISC. To confirm that this effect is mediated by the MAP kinase pathway, we analyzed DISC formation under conditions in which this pathway was either inhibited by PD 98059, the MEK inhibitor, or activated by the expression of $MEK1^{Q56P}$. TRAIL–TRAIL-receptor complexes were immunoprecipitated from TRAIL-treated cells by use of an antibody to polyhistidine. As shown in Fig. 6*D*, comparable amounts of FADD coimmunoprecipitated with TRAIL receptors from control, Ras-transformed, or $MEK1^{Q56P}$-expressing cells. In contrast, significantly more caspase 8 was coimmunoprecipitated with TRAIL receptors from Ras-transformed cells than from control cells. Pretreatment of Ras-transformed cells with the farnesyl transferase inhibitor SCH 66336 or with the MEK inhibitor PD 98059 reduced the amounts of coimmunoprecipitated caspase 8. The expression of constitutively active MEK increased the recruitment of caspase 8 to TRAIL receptors almost as efficiently as did oncogenic Ras. These results indicate that the observed enhancement of TRAIL receptor DISC formation in Ras-transformed cells is mediated by the MAP kinase pathway.

DISCUSSION

Although selective killing of cancer cells by TRAIL has been reported in many studies (1), the molecular mechanisms behind this selectivity remain unclear. Research has been focused on a comparison of apoptotic signals induced by TRAIL in normal cells *versus* those obtained from cancer patients. These studies implicated several proteins, including the protease-deficient homolog of caspase 8, FLIP (25), and the TRAIL decoy receptors DcR1 and DcR2 (26), in resistance of normal cells to TRAIL. However, a firm correlation between the cellular expression of these proteins and TRAIL resistance has not been established (27, 28).

Given the number of genetic alterations that occur during neoplastic transformation in humans (8), it is difficult to single out individual changes that are responsible for the acquisition of the TRAIL-sensitive phenotype. Here we describe the use of genetically defined transformation to investigate how conversion of human cells from normal to tumorigenic renders them sensitive to TRAIL-induced apoptosis. This experimental model mimics the stepwise accumulation of genetic alterations that occurs in human cancers: inactivation of tumor suppressors, acquisition of an unlimited life span, and receipt of a continuous mitogenic signal (9). Both normal and immortalized cells were found to be resistant to TRAIL-mediated apoptosis, indicating that premalignant changes, including inactivation of tumor suppressors and acquisition of an unlimited life span, are not sufficient to sensitize cells to TRAIL. However, the subsequent conversion of immortalized cells to tumorigenic ones by activated Ras rendered them susceptible to TRAIL-mediated apoptosis.

Ras-mediated sensitization of cells to TRAIL-induced apoptosis may involve at least two mechanisms. First, oncogenic Ras upregulates the levels of one of the TRAIL receptors, DR5. Second, transformation of cells with Ras appears to facilitate recruitment of caspase 8 to TRAIL DISC. Because the overall amounts of FADD associated with TRAIL receptors was not increased on Ras transformation, it is likely that activated Ras somehow facilitates the interaction of caspase 8 with FADD. This process may involve several mechanisms. For example, one can speculate that the Ras-induced MAP kinase pathway may trigger direct phosphorylation/dephosphorylation of caspase 8, thereby affecting its FADD-binding function. Alternatively, the MAP kinase pathway may down-regulate the expression of a protein that competes with caspase 8 for FADD binding. Obviously, additional studies are needed to determine the exact mechanism of this sensitization.

Because Ras is capable of inducing genomic instability (29), it might, in principle, sensitize cells to TRAIL by promoting irreversible genetic alterations that would disable certain antiapoptotic pathways. Two observations make this scenario unlikely. First, the Ras-transformed cells used in our studies were found to be polyclonal (9). Second, the proapoptotic effects of Ras could be reversed by inhibitors of either farnesyl transferase or MEK. Apparently, continuous Ras signaling is essential for Ras-transformed cells to maintain both the transformed phenotype (30) and sensitivity to TRAIL. Alternatively, the activity of the farnesylation inhibitor could be related to its ability to regulate RhoB (31), suggesting that regulation of other small GTP-binding proteins may be important in regulating the sensitivity to TRAIL.

Our results indicate that the proapoptotic effect of Ras is mediated by the MAP kinase pathway. It has been reported recently that activation of this pathway suppresses TRAIL-induced apoptosis in HeLa cells (32). One possible explanation for this apparent discrepancy is that MAP kinase is capable of eliciting both proapoptotic (24, 33, 34) and prosurvival responses (35). The antiapoptotic effect of MAP kinase appears to be cell type specific. For example, activation of MAP kinase by phorbol myristate acetate was shown to suppress receptor-mediated apoptosis in some, but not all, types of cells (36). A prosurvival effect of the MAP kinase pathway has, at least in part, been attributed to ERK-dependent up-regulation of antiapoptotic FLIP proteins (37). However, positive regulation of FLIP expression by the MAP kinase pathway has been observed only in a limited set of human cells (38). Likewise, we did not observe any significant changes in the levels of FLIP proteins either on activation of MAP kinase by Ras or constitutively activated MEK (Fig. 6*B*), or on treatment of Ras-transformed BJ and HEK cells with MEK inhibitors (data not shown).

In summary, using human cells that were progressively converted from normal into tumorigenic we demonstrated that (*a*) premalignant changes, including inactivation of tumor suppressors and immortalization, are not sufficient to sensitize human cells to TRAIL; (*b*), transformation of the immortalized cells with the growth-promoting oncogene H-*ras*-V12 renders them susceptible to TRAIL-induced apoptosis; (*c*) oncogenic Ras potentiates TRAIL-induced recruitment and activation of the initiator caspase 8; (*d*) the proapoptotic effects of Ras are reversible and involve the MAP kinase pathway; and (*e*) constitutive activation of MAP kinase sensitizes immortalized human cells to TRAIL. Because aberrant activation of MAP kinases is often associated with a neoplastic phenotype (19), sustained MAP kinase activity may potentially serve as an indicator of malignant transformation recognized by a TRAIL-based antitumor surveillance system.

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The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim

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Abstract

Previously, we showed that the proteasome inhibitor bortezomib/Velcade (formerly PS-341) synergizes with the protein tumor necrosis factor α–related apoptosis-inducing ligand (TRAIL), a ligand for certain death receptors, to induce apoptosis in cell lines derived from prostate and colon cancers. Because apoptosis is often triggered by BH3-only proteins of the Bcl-2 family, we have explored the hypothesis that bortezomib contributes to the apoptosis by up-regulating their levels. Indeed, bortezomib induced increases of Bik and/or Bim in multiple cell lines but not notably of two other BH3-only proteins (Puma and Bid) nor other family members (Bax, Bak, Bcl-2, and Bcl-x_L). The increase in Bik levels seems to reflect inhibition by bortezomib of its proteasome-mediated degradation. Importantly, both Bik and Bim seem central to the proapoptotic function of bortezomib because mouse embryo fibroblasts in which the genes for both Bik and Bim had been disrupted were refractory to its cytotoxic action. Similarly, the synergy between bortezomib and TRAIL in killing human prostate cancer cells was impaired in cells in which both Bik and Bim were down-regulated by RNA interference. Further evidence that bortezomib acts through the mitochondrial pathway regulated by the Bcl-2 family is that deficiency for APAF-1, which acts downstream of Bcl-2, also blocked its apoptotic effect. These results implicate BH3-only proteins, in particular both Bik and Bim, as important mediators of the antitumor action of bortezomib and establish their role in its enhancement of TRAIL-induced apoptosis. [Mol Cancer Ther 2005;4(3):443–9]

Introduction

The proteasome inhibitor bortezomib/Velcade (formerly PS-341) has recently entered clinical practice as a treatment for multiple myeloma and is undergoing clinical trials for other types of cancer (1). Its mode of action is not established but is very likely to involve promotion of apoptosis (2, 3). Diverse mechanisms have been proposed. Some results suggest that bortezomib might act through the ''death receptor'' pathway, in which extracellular ligands promote apoptosis through the activation of caspase-8 (4). Gene expression studies in cultured cells have shown that bortezomib treatment increases the level of the mRNAs for a number of proapoptotic proteins, including that of the death receptor DR5, which can be engaged by the tumor necrosis factor α–related apoptosis-inducing ligand (TRAIL; refs. 5, 6). Moreover, bortezomib has been reported to reduce levels of c-FLIP (7), which counters the activation of caspase-8, or to increase the activation of caspase-8 and its target Bid through mechanisms not involving c-FLIP (6). On the other hand, other results implicate the intrinsic pathway to apoptosis, in which the Bcl-2 protein family and mitochondria play key roles (8). Thus, bortezomib has been reported (a) to stabilize and activate the tumor suppressor p53 (9), which acts upstream of Bcl-2; (b) to stabilize IκB and thereby decrease the antiapoptotic effects of nuclear factor κB (10), which are frequently mediated through the Bcl2 family; and (c) to damage mitochondria through generation of reactive oxygen species, a response attenuated by Bcl-2 (11).

Such findings favor the view that bortezomib and perhaps other modes of proteasome inhibition promote apoptosis at least in part through the pathway regulated by the Bcl-2 family (2, 3). As well as members that promote cell survival (e.g., Bcl-2 and Bcl-x_L), this family includes two proapoptotic groups: the eight or more proteins termed ''BH3-only'' because they bear only the small BH3 protein-interaction domain (e.g., Bik, Bim, Bid, and Puma) serve as triggers for the apoptotic signal, whereas Bax and Bak act downstream to impose apoptosis, probably mainly through permeabilization of mitochondria (8, 12, 13). We showed previously (6) that bortezomib synergizes with TRAIL to induce apoptosis in prostate and colon cancer cell lines. Pertinently, mitochondrial permeabilization was

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implicated by the early release of cytochrome *c* and second mitochondrial activator of caspases, proteins that promote the activation of caspase-9 via the scaffold protein Apaf-1, or antagonize the inhibitory effect of XIAP on caspase-9 respectively. Furthermore, in mouse embryonic fibroblasts (MEF), Bax and Bak were required for the apoptosis induced by bortezomib alone, and the absence of Bak protected cells against death induced by bortezomib combined with TRAIL (6).

As TRAIL has promise as an anticancer agent (4), we have explored further how bortezomib sensitizes cells to its action. Because the level of BH3-only proteins often seems a critical determinant of whether apoptosis ensues (8, 12, 14), our previous findings (6) have stimulated us to test whether bortezomib might act by increasing the level of certain BH3-only proteins. In keeping with that hypothesis, we report here that bortezomib induces increased levels of the BH3-only proteins Bik and Bim in a number of cancer cell lines and in MEFs. Bim is known to be required for apoptosis induced in hematopoietic cells by several types of cytotoxic stimuli and to participate in the developmentally programmed death of several cell types (12, 14, 15). Less is known about Bik, but the mouse gene (previously denoted Blk) is expressed in diverse cell types (16), including some cancer cell lines (17), and Bik mutations have been reported in some human B cell lymphomas (18). Significantly, we show that suppression of expression of both these proteins in MEFs or prostate cancer cells inhibits the apoptosis induced by bortezomib or by combined bortezomib/TRAIL treatment.

Materials and Methods

Cell Lines and Reagents

MEFs, Du145, PC-3, and Alva human prostatic cancer cell lines, 293T, and MCF-7 human breast cancer cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA), 100 units/mL penicillin, and 100 μg/mL streptomycin. The human prostatic cancer cell line LNCaP was grown in RPMI 1640 (Invitrogen) with glutamine and the same supplements. Cultures were maintained at 37°C at 95% humidity. Antibodies were obtained from the following sources: Bik from Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-2, Bax, and Bak from Upstate Biotechnology (Lake Placid, NY); Bim from Stressgen Bioreagents (Victoria, British Columbia, Canada); poly(ADP-ribose) polymerase from Trevigen (Gaithersburg, MD); glyceraldehyde 3′-phosphate dehydrogenase from Chemicon (Temecula, CA); and HA, FLAG, and FLAG agarose from Sigma-Aldrich Co. (St. Louis, MO). Recombinant human TRAIL was prepared as described previously (6). Bortezomib (Velcade, formerly PS-341) was a gift of Millenium, Inc. (Cambridge, MA).

Cell Viability Assays

Assays employing 4′,6-diamido-2-phenylindole (DAPI) were done as described previously (6). Assays measuring cell survival were done as follows: cells were plated in 6- or 24-well plates. Following an 18-hour treatment with bortezomib and/or TRAIL, nonviable cells were aspirated from the plates and the remaining attached cells rinsed twice with PBS. Depending on the cell density, plates were treated in either of two ways: (a) the cells were trypsinized, and cell number determined by hemacytometer; or (b) the cells were fixed on the plates with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes, followed by two rinses with PBS. The cells in a series of adjacent fields from the same region of each well were then counted microscopically. Where possible, both methods were used and equivalent results were obtained.

Plasmids and Transfection

Human Bik was cloned from cDNA that had been reverse transcribed from LNCaP cell total RNA, using as 5′ primer 5′-CCATGGATCCACCATGGACTACAAGGACGACGATGACAAGATGTCTGAAGTAAGACCCCTCTCC, which contains the sequence coding for the FLAG epitope tag and 3′ primer 5′-AGTAGTGCGGCCGCTCACTTGAGCAGGTGCAGG. Integrity of the constructs was verified by DNA sequencing (University of Colorado Cancer Center DNA Sequencing and Analysis Core). The constructs expressing RNA interference (RNAi) directed against Bik from the human U6 promoter (target sequence 5′-CATAATGAGGTTCTGGAGA), and control RNAi, were made using the Silencer Express kit (Ambion, Austin, TX) and inserted into the pcDNA3 vector (Stratagene, La Jolla, CA), following excision of sequences for the cytomegalovirus promoter. The construct expressing RNAi against Bim was generated in the pSuper vector as described (19). It contains the sequence 5′-TGATGTAAGTTCTGAGTGGTG, which is common to all known Bim mRNA isoforms and is 100% conserved in human Bim. A hygromycin resistance cassette was added to this construct for generating stable cell lines. Transient transfections were done with Effectene (Qiagen, Valencia, CA) following the manufacturer's instructions. Stable cell lines were derived by G418 (anti-Bik, LNCaP) or hygromycin (anti-Bim, MCF-7) selection.

Reverse Transcription-PCR

cDNA was synthesized from total RNA (20) with SuperScript II and random primers (Invitrogen) following the manufacturer's instructions. Primers for Bik were 5′-GGAGACCCTCCTGTATGAGC (forward) and 5′-ACCTGTTCGCAGGACACC (reverse) and were designed with Primer3 software (21).

Immunoprecipitation

Immunoprecipitation of Bik was done as follows: cells were lysed in >5 volumes of lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 20 mmol/L EDTA, 20 mmol/L NaF, 0.5% Igepal (NP40), 40 mmol/L β-glycerophosphate, 0.2 mmol/L phenylmethylsulfonylfluoride, and protease inhibitor cocktail (Sigma)]. Lysates were rotated overnight at 4° with either agarose beads to which anti-FLAG epitope antibody had been covalently coupled (Sigma), or protein-A-protein G agarose beads (Calbiochem/EMD Biosciences, San Diego, CA) to which

anti-Bik antibody had been bound by incubation. After extensive rinses with lysis buffer, bound proteins were eluted with 100 mmol/L glycine (pH 2.3), neutralized, and processed for Western blotting.

Cell Sorting and Western Blotting

Cells were sorted on the basis of green fluorescent protein (GFP) expression at the University of Colorado Cancer Center Flow Cytometry Core. Western blotting was done as described (6).

Mouse Embryo Fibroblasts

Fibroblasts were prepared from mouse embryos in which the genes for Bim, Blk, or Apaf-1 had been disrupted by homologous recombination (9, 16). Fibroblasts doubly deficient in Bim and Blk were obtained from embryos obtained from crosses between homozygous *bim* and *bik*-deficient mice.

Statistics

Data were analyzed first by ANOVA. Pairwise comparisons were then done with Bonferroni *t* test (22).

Results

Regulation of the Level of BH3-Only Proteins by Bortezomib

We showed previously that bortezomib treatment of LNCaP cells did not affect the levels of either Bax or Bak (3). To examine the possibility that certain proteins of the BH3-only subfamily might be up-regulated by bortezomib, we probed Western blots from a panel of seven cell lines including prostate, colon, and breast cancer cell lines with antibodies directed against diverse proteins of the Bcl-2 family (Fig. 1A). Except for a slight rise in Bid in two lines (HC-4 and MCF-7), bortezomib treatment did not affect the levels of Bcl-xL, Bcl-2, Bax, Bak, or PUMA. In striking contrast, the level of the BH3-only protein Bik was elevated by the drug treatment in every cell line examined. Moreover, the level of Bim rose in HC-4 colon cancer cells (Fig. 1A), MEFs (Fig. 1C), and MCF-7 human breast cancer cells (Fig. 1A). The fold changes varied among cell lines but was as high as 4.2-fold in Du145 cells. The kinetics of induction of Bik in LNCaP cells (Fig. 1B) revealed that significant elevation was evident after only 1 hour of exposure to bortezomib, and the level continued to increase for several hours, indicating that Bik might well have a role in the induction of apoptosis. Rapid elevation was also observed in HC-4 and PC-3 cells (data not shown). As measured by the number of cells occupying the sub-G_1 peak on fluorescence-activated cell sorting, 17 hours of bortezomib treatment induced relatively little cell death in these lines (LnCaP, 8.8% increase in cell death when compared with control untreated cells [all data is the average of triplicate determinations]; MCF-7, 0%; 293T, 1%; PC-3, 6.71%; HC-4, 3.16%; Du145, 1%). Longer incubations killed significantly more cells in all lines.

To determine whether Bik up-regulation instead reflected inhibition of proteasome-mediated protein degradation (3), we tested whether ubiquitinated forms of the protein accumulated in response to bortezomib. To do so, a FLAG-Bik expression construct was transfected into 293T cells together with a HA-tagged ubiquitin expression construct. Bik was then immunoprecipitated from the transfected cells, subjected to electrophoresis and shown by Western blotting using antibodies directed against HA and Bik. Figure 2 shows that both antibodies detected higher molecular weight bands specifically in Bik-transfected cells. The increase in their intensity following bortezomib treatment suggests that Bik is normally degraded by the proteasome via the standard ubiquitin-mediated pathway. Very little HA-specific staining was detected at the molecular weight expected for monoubiquitinated Bik ~28 kDa. As suggested by others (23), this result could reflect the preferential utilization of monoubiquitinated proteins in signaling pathways distinct from degradation.

Thus, bortezomib seems to up-regulate Bik by inhibiting its proteasomal degradation, and recent work suggests that the abundance of Bim can also be regulated by this mechanism (24, 25).

Central Roles of Bik and Bim in Mediating Apoptosis by TRAIL and Bortezomib

We showed previously (6) that bortezomib treatment increased sensitivity to TRAIL-induced apoptosis in LNCaP prostate cancer cells, HC-4 Bax-negative colon cancer cells, and MEFs. MEFs containing both Bax and Bak undergo apoptosis when treated with bortezomib alone. As

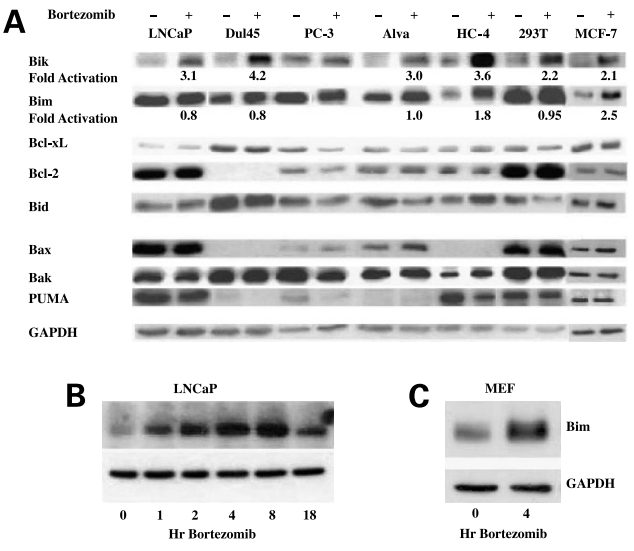

Figure 1. Effects of bortezomib treatment on the levels of members of the Bcl-2 protein family. **A,** the levels of Bcl-2, Bcl-xL, Bax, Bak, PUMA and Bik, Bid, and Bim proteins were assessed in the indicated cell lines. Cells were incubated with 1 μmol/L bortezomib or vehicle for 17 h, lysed, and prepared for Western blot analysis with the indicated antibodies. To quantitate these Western blot bands, regions of interest were defined around the bands. Kodak ID software was used to measure the sum intensity in these regions. The fold-activation reported was determined by dividing the value obtained from the treated cells by those for the control untreated. **B,** LNCaP cells were incubated with 1 μmol/L bortezomib for the indicated times and processed for Western blotting for Bik and GAPDH as a loading control. **C,** MEF were incubated with 1 μmol/L bortezomib for 4 h and processed for Western blotting for Bim and GAPDH as a loading control.

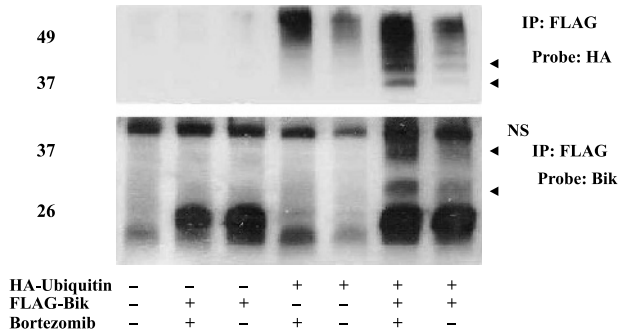

Figure 2. Regulation of Bik protein levels by bortezomib. 293T cells were transfected with expression constructs for HA-ubiquitin and FLAG-Bik in the indicated combinations. The transfected cells were treated with 1 μmol/L bortezomib for 24 h. Lysates of the cells were incubated with anti-FLAG antibody coupled to agarose beads and bead eluates processed for Western blotting as described in Materials and Methods. *Top,* probed with antibody to the HA-tagged ubiquitin. *Arrows,* bands (most prominent in bortezomib-treated cells) specific to cells expressing both the Bik and ubiquitin constructs. *Bottom,* probed with antibody to Bik. Nonspecific band appearing in all preparations (*NS*). *Arrows,* bands specific to cells expressing both the Bik and ubiquitin constructs.

reviewed elsewhere (8, 12), it is well established that overexpression of BH3-only proteins such as Bim and Bik promotes apoptosis (e.g., refs. 26–28). To determine whether the elevated levels of Bik and/or Bim described above contributed to TRAIL sensitivity or to bortezomib-induced apoptosis, we have taken two genetic approaches. To analyze the cancer cell lines, we have generated derivative lines in which Bik or Bim expression has been down-regulated by RNAi via the synthesis of small hairpin RNAs (shRNA; ref. 29). For the MEFs, we have analyzed cells bearing homozygous disruptions of *bim* (15), *bik* (16), or both genes.

Because Bim was not induced by bortezomib in the prostate cancer cell lines examined, we evaluated other malignant cell lines and found significant induction of Bim by bortezomib treatment in MCF-7 cells (Fig. 1A). MCF-7 breast cancer cells were stably transfected with a construct expressing a shRNA directed against Bim. Figure 3A (*top*) shows that the elevation of Bim levels in wild-type cells by bortezomib was almost entirely suppressed by RNAi. The effect seems to be specific, because induction of Hsp-70 by bortezomib was unaffected, whereas Bcl-2 levels remained stable (Fig. 3A), and a point mutation in the hairpin used ablates the suppression by this RNAi.[3] A preliminary experiment using nuclear DAPI uptake as a marker for apoptosis suggested that bortezomib-induced apoptosis in the cell line expressing RNAi did not differ significantly from wild-type MCF-7 after 24 hours (Fig. 3A, *bottom*). To evaluate further the effect of lowering Bim on the ability of bortezomib to enhance TRAIL-mediated apoptosis, we counted the cells remaining attached to the culture dish after combined treatment as an assay for cell survival. The cell line expressing Bim RNAi remained sensitive to TRAIL (Fig. 3B).

[3] P. Bouillet et al., unpublished results.

Similar experiments were conducted using LNCaP prostate cancer cells stably transfected with a shRNA construct directed against Bik. As shown in Fig. 3C, the bortezomib-induced elevation of Bik levels was strongly suppressed in these cells. However, there was no qualitative change in the extent of poly(ADP-ribose) polymerase cleavage resulting from treatment with TRAIL, or the combination of bortezomib and TRAIL, at the concentrations used. Nevertheless, RNAi against Bik can enhance cell survival (see below).

Unlike LNCaP cells, MEFs can be killed by bortezomib alone, as well as by the drug combination. Therefore, the MEFs derived from mice rendered deficient in Bim, Bik, or both proteins by homologous recombination (15, 16) provided an independent approach for evaluating whether either protein was essential for the apoptosis elicited by the drugs. Wild-type and mutant MEFs were treated with bortezomib, TRAIL, or the combination for 18 hours. Cells were then detached from the plates with trypsin and scored for the uptake of DAPI by fluorescence microscopy (Fig. 4). Notably, in the response to bortezomib alone, the MEFs deficient in both Bim and Blk showed <60% of the apoptosis given by the wild-type cells ($P < 0.001$). With that stimulus alone, even the MEFs lacking only Bik showed significantly less death than the wild-type cells ($P < 0.01$), but the absence of Bim alone seemed to have no statistically significant effect. With both bortezomib and TRAIL, apoptosis was high with the cells of all the genotypes, and although the extent seemed slightly lower in the MEFs lacking both Bim and Bik, the decrease was not statistically significant. Overall, the data derived from MEF, MCF-7, and LNCaP cells suggested that, although antiapoptotic effects of suppressing expression of Bim or Bik alone might be difficult to detect, suppression of both together might well convey significant protection. We tested this idea in LNCaP cells by using RNAi to down-regulate expression of both proteins. Control LNCaP cells (expressing an shRNA construct with no effect on Bik levels) and the LNCaP cell line stably expressing a shRNA directed against Bik were transiently transfected with a construct expressing either GFP alone, or GFP plus the anti-Bim shRNA shown to be effective in MCF-7 cells. Twenty-four hours later, GFP-positive cells were isolated by fluorescence-activated cell sorting and plated. After a recovery time of 10 to 12 hours, the cells were treated with bortezomib plus graded concentrations of TRAIL for 12 to 18 hours, then assayed for apoptosis using detachment as a marker. A representative experiment is shown in Fig. 5A. At all TRAIL concentrations tested, the cells expressing shRNAs against both Bim and Bik showed significantly greater survival than those expressing each single shRNA ($P < 0.05$ at 5 ng/mL TRAIL). Moreover, at the highest TRAIL concentration (100 ng/mL), inhibiting expression of either Bim or Bik alone also provided significant protection. However, because most cells were undergoing apoptosis at this concentration of TRAIL, no differences in the extent of poly(ADP-ribose) polymerase cleavage were evident (cf. Fig. 3C).

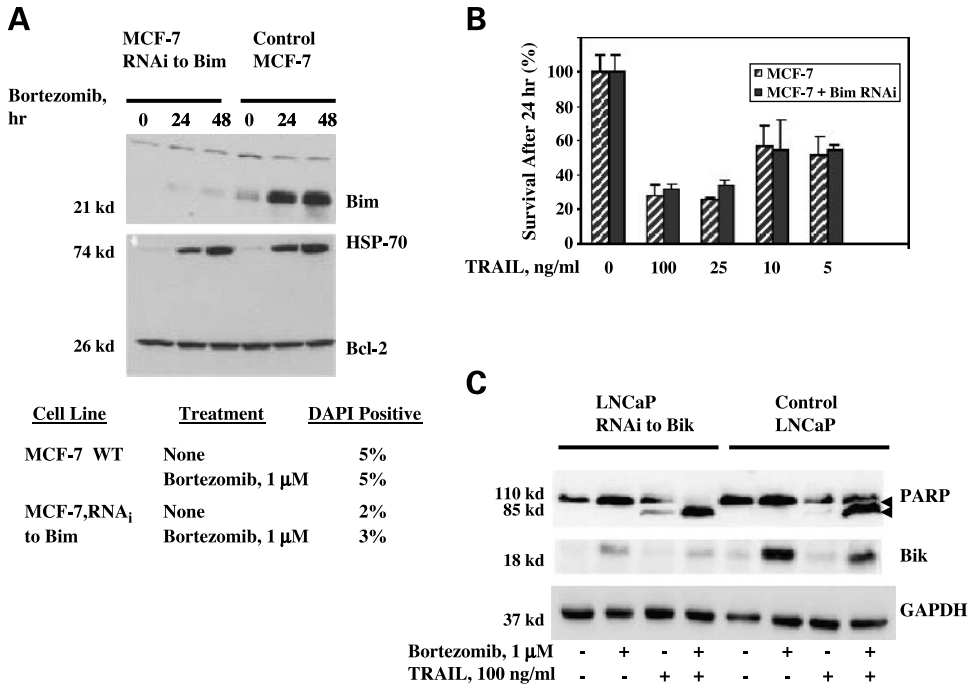

Figure 3. Effectiveness of RNAi directed against Bim and Bik in stable cell lines. **A,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 μmol/L bortezomib for the indicated times. Cell lysates were analyzed by Western blotting for the indicated proteins. *Bottom,* Cells treated with 1 μmol/L bortezomib for 24 h were scored for nuclear DAPI uptake as described in Materials and Methods. **B,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 μmol/L bortezomib and the indicated TRAIL concentrations for 24 h. Surviving cells were scored as described in Materials and Methods. **C,** LNCaP human prostatic cancer cells stably expressing a shRNA against Bik, and the parental cell line, were treated with bortezomib and TRAIL as indicated for 6 h. Cell lysates were analyzed for the indicated proteins by Western blotting.

Taken together, these results on BH3-only proteins suggest that the mitochondrial pathway of cell death plays a critical role in the ability of bortezomib to sensitize cells to TRAIL-induced cell death. If so, the proapoptotic effect should be suppressed by elimination of a critical downstream effector of mitochondrial disruption, such as the caspase-9 activator Apaf-1. To evaluate this, we studied the response of MEFs deficient in Apaf-1 to these agents (Fig. 5B). Clearly, the absence of Apaf-1 markedly decreased the ability of bortezomib to kill MEFs. At the TRAIL dose used in these studies (1 μg/mL), there was also a small but statistically significant ($P < 0.05$) decrease in the sensitivity of the cells to the combination treatment. This result supports the notion that both the intrinsic and extrinsic pathways are important for TRAIL-mediated apoptosis in MEFs.

Discussion

In this paper, we have shown that bortezomib treatment of a number of cancer cell lines induces increases in the levels of the BH3-only proteins Bik and Bim. Moreover, suppression of this induction, particularly of Bik, results in increased resistance of the cells to apoptosis caused by either bortezomib alone (for MEFs) or, in the case of the LNCaP cells, which are not killed by bortezomib alone, by the combination of TRAIL plus bortezomib. In addition, we found that concomitant suppression of Bim and Bik in both MEFs and LNCaP cells resulted in significantly more resistance than suppression of Bim or Bik individually.

More than eight BH3-only proteins have been described thus far in mammalian cells (8), and gene targeting has been used to clarify the apoptotic roles of six of them: Bim (15), Bad (30), Bid (31), Bik (16), Puma, and Noxa (32). Developmental lesions in such knockout mice range from dramatic (Bim) to as yet undetected (Bik). It seems likely, as is the case for Bax and Bak, that the functions of some BH3-only proteins overlap and hence that specific roles for such proteins appear only when more than one of the genes is suppressed. For example, we have recently found that male mice deficient in both Bim and Bik are sterile,[4] whereas animals deficient in Bim or Bik alone have normal fertility. In accord with redundant function, we observed greatest resistance to apoptosis when expression of both Bim and Bik expression was suppressed.

As yet it is not entirely clear how BH3-only proteins activate the apoptotic machinery or how much their functions overlap (13). There is wide agreement that association of BH3-only proteins with antiapoptotic Bcl-2 family members is a critical step, and that the relative level of these opposing factions is important. In response to bortezomib treatment, we found that levels of Bcl-2 and Bcl-x_L did not change (Fig. 1), although Mcl-1 levels were elevated (data not shown), in accord with its reported regulation by the proteasome (33). It is possible that the binding affinities of Bcl-2 prosurvival family members to various BH3-only proteins varies widely, and hence that the response to an apoptotic stimulus depends upon the precise cellular composition of these two factions (34). In addition to binding prosurvival family members and thus

[4] L. Coultas et al., unpublished results.

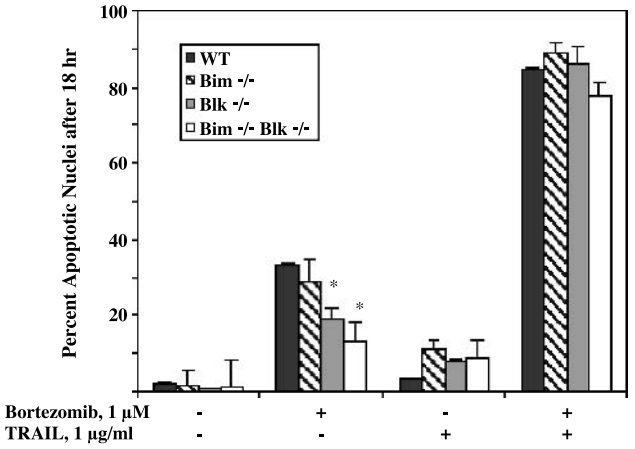

Figure 4. Bortezomib and TRAIL-mediated apoptosis in Bim and/or Bik-deficient MEFs. MEFs were treated as indicated for 18 h and scored for nuclear DAPI uptake as described in Materials and Methods. *Columns,* mean of nuclei and cell counts from two independent experiments; *bars,* SD. *, $P < 0.01$, statistically significant deviation from wild-type value.

releasing their inhibition of Bax and Bak activation, it has been proposed that certain BH3-only proteins, such as Bid (35), can directly interact with Bax and Bak to promote apoptosis.

Preliminary experiments using reverse transcription-PCR and Bik promoter-CAT constructs suggested that bortezomib did not augment Bik transcription in LNCaP or HC-4 cells (data not shown). Our demonstration that Bik is ubiquitinated (Fig. 2) suggests that its rapid accumulation in many cell lines in response to bortezomib is due to stabilization consequent to proteasome inhibition. Others have reported accumulation of Bik in the presence of other proteasome inhibitors (27, 36). Of interest, in one study Bik accumulated to much higher levels in cell lines which overexpressed Bcl-x_L, suggesting that Bcl-x_L served to sequester Bik (27). We observed no correlation, however, between the level of Bcl-2 or Bcl-x_L and the level of Bik induced by bortezomib (cf. Fig. 1A).

Importantly, we showed that MEFs deficient in Bik or both Bim and Bik were significantly resistant to bortezomib-induced apoptosis (Fig. 4). Similarly, LNCaP prostate cancer cells in which both Bim and Bik had been suppressed were resistant to TRAIL-induced apoptosis in the presence of bortezomib (Fig. 5A). We showed previously (6) that enhanced cleavage of caspase-8 and Bid contributed to the synergy between bortezomib and TRAIL in inducing apoptosis. The present results suggest that an additional mechanism is bortezomib-mediated increases of the BH3-only proteins Bik or Bim. When these proteins reach a threshold level, they presumably can neutralize antiapoptotic proteins of the Bcl-2 family and thereby allow activation of Bax and/or Bak, leading to permeabilization of mitochondria and activation of caspase-9 (8). This mechanism is consistent with the marked potentiation of bortezomib on the apoptotic action of drugs such as doxorubicin (37). Thus, bortezomib apparently can promote apoptosis through both this pathway and that involving enhanced Bid cleavage. The relative importance of the intrinsic and extrinsic pathways in its action may

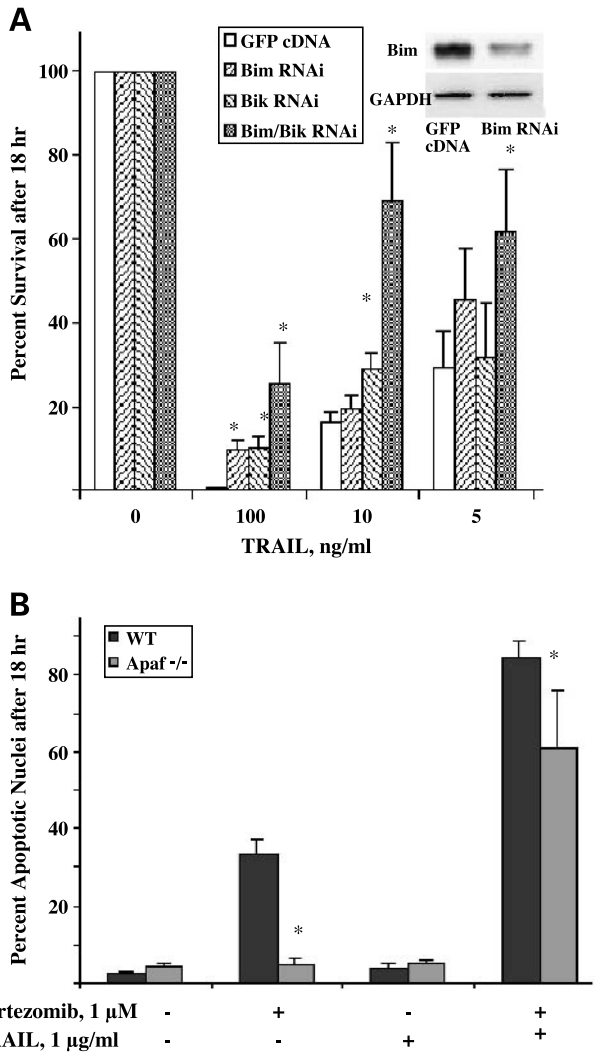

Figure 5. A, Protection against TRAIL-mediated apoptosis by RNAi directed against Bim and/or Bik. LNCaP human prostatic cancer cells stably expressing a shRNA directed against Bik were transiently transfected with constructs expressing GFP and a shRNA directed against Bim, or the cDNA encoding GFP alone. As a control, LNCaP cells stably expressing an RNAi with no effect on Bik or Bim levels were transiently transfected with constructs expressing the cDNA for GFP and or an shRNA directed against Bim, or the cDNA for GFP alone. Cells expressing GFP were sorted by FACS, plated, and treated with TRAIL at the indicated concentrations in the presence of 1 μmol/L bortezomib for 18 h. Cell survival was scored as described in Materials and Methods. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistically significant deviation from control (GFP) values. *Inset,* Western blot of control LNCaP cells transiently transfected with the constructs expressing GFP and RNAi directed against Bim. GFP-positive and GFP-negative cells were sorted by FACS, lysed, and analyzed for Bim expression by Western blot using GAPDH as a loading control. **B,** bortezomib and TRAIL-mediated apoptosis in APAF-1-deficient MEFs. MEFs were treated as indicated for 18 h and scored for nuclear DAPI uptake as described in Materials and Methods. *Columns,* mean of nuclei and cell counts from two independent experiments; *bars,* SD. *, $P < 0.05$, statistically significant deviation from wild-type value.

well vary with cell type. In any case, these findings and those we reported previously (6) provide the rational for further exploration of the potential of combining bortezomib and TRAIL in cancer therapy.

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